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VEGF-mediated vascular functions in health and disease

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並倚靠他，他就必成全。

詩篇三十七篇五節

VEGF-MEDIATED VASCULAR FUNCTIONS IN HEALTH AND DISEASE

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Ziquan Cao

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TABLE OF CONTENTS

ABSTRACT 1

LIST OF PAPERS 4

RELATED PUBLICATIONS 5

ABBREVIATIONS 6

INTRODUCTION 9

AIMS OF THIS THESIS 33

METHODS 35

RESULTS AND DISCUSSIONS..... 39

CONCLUSIONS 50

FUTURE PERSPECTIVES..... 51

ACKNOWLEDGEMENTS 53

REFERENCES..... 56

ABSTRACT

Angiogenesis is essential for physiological processes including embryonic development, tissue regeneration, and reproduction. Under various pathological conditions the same angiogenic process contribute to the onset, development, and progression of many human diseases including cancer, diabetic complications, ocular disease, chronic inflammation and cardiovascular disease. Vascular endothelial growth factor (VEGF) is a key angiogenic factor for physiological and pathological angiogenesis. In addition to its strong angiogenic activity, VEGF also potently induces vascular permeability, often causing tissue edema in various pathological tissues. VEGF transduces its vascular signal through two tyrosine kinase receptors-VEGFR1 and VEGFR2, the latter being a functional receptor that mediates both angiogenic and vascular permeability effects. To study physiological and pathological functions of VEGF, we developed novel zebrafish disease models that permit us to study hypoxia-induced retinopathy and cancer metastasis processes. We have also administered anti-VEGF and anti-VEGFR specific antibodies to healthy mice to study the homeostatic role of VEGF in the maintenance of vascular integrity and its functions in various tissues and organs. Finally, using a zebrafish model, we evaluated if VEGF expression is regulated by circadian clock genes. In paper I, we developed protocols that create hypoxia-induced retinopathy in adult zebrafish. Adult *fli1:EGFP* zebrafish were placed in hypoxic water for 3-10 days with retinal neo-vascularization being analyzed using confocal microscopy. This model provides a unique opportunity to kinetically study the development of retinopathy in adult animals using non-invasive protocols and to assess the therapeutic efficacy of orally administered anti-angiogenic drugs. In paper II, we developed a zebrafish metastasis model to dissect the complex events of hypoxia-induced tumor cell invasion and metastasis in association with angiogenesis at the single-cell level. In this model, fluorescent DiI-labeled human or mouse tumor cells were implanted into the perivitelline cavity of 48-hour-old zebrafish embryos, which were subsequently placed in hypoxic water for 3 days. Tumor cell invasion, metastasis and pathological angiogenesis were analyzed using fluorescent microscopy in the living fish. The average experimental time for this model is 7 days. Our protocol offers an opportunity to study molecular mechanisms of hypoxia-induced cancer metastasis. In paper III, we show that systemic delivery

of an anti-VEGF or an anti-VEGF receptor (VEGFR)-2 neutralizing antibody cause global vascular regression in mice. Among all examined tissues, the vasculature in endocrine glands, intestinal villi, and the uterus are most affected in response to VEGF or VEGFR-2 blockades. Pro-longed anti-VEGF treatment resulted in a significant decrease in the circulating levels of the predominant thyroid hormone, free thyroxine, but not the minimal isoform of triiodothyronine, suggesting that chronic anti-VEGF treatment impairs thyroid function. These findings provide structural and functional bases of anti-VEGF-specific drug-induced side effects in relation to vascular changes in healthy tissues. In paper IV, we show that disruption of the circadian clock by constant exposure to light coupled with genetic manipulation of key genes in the zebrafish led to impaired developmental angiogenesis. A *bmal1*-specific morpholino inhibited developmental angiogenesis in zebrafish embryos without causing obvious nonvascular phenotypes. Conversely, a *period2* morpholino accelerated angiogenic vessel growth, suggesting that *Bmal1* and *Period2* display opposing angiogenic effects. These results offer mechanistic insights into the role of the circadian clock in regulation of developmental angiogenesis, and our findings may be reasonably extended to other types of physiological or pathological angiogenesis. Overall, the results in this thesis provide further insight to angiogenic mechanistic properties in tissues and suggest possible novel therapeutic targets for the treatment of various angiogenesis-dependent diseases.

Blodkärlsnybildning, så kallad angiogenes, är viktigt för fysiologiska processer vid embryonal utveckling, vävnadsregenerering och reproduktion. Samma angiogena process kan också under olika sjukdomstillstånd bidra till uppkomst, utveckling och progress av många sjukdomar, såsom cancer, diabeteskomplikationer, ögonsjukdomar, kronisk inflammation samt hjärt-kärlsjukdom. Vascular endothelial growth factor (VEGF) är mycket viktig för fysiologisk och patologisk angiogenes. Utöver sin starka angiogena effekt inducerar VEGF även ökad kärlpermeabilitet, som ofta orsakar ödem. VEGF utövar sin effekt på kärlen via två tyrosinkinasreceptorer: VEGFR1 och VEGFR2, där den senare är en funktionell receptor som förmedlar både angiogena signaler och har effekter på vaskulär permeabilitet. För att öka möjligheterna att studera fysiologiska och patologiska funktioner av VEGF, har vi utvecklat sjukdomsmodeller i zebrafisk - hypoxi-inducerad retinopati och metastasering av cancer. Vi har också givit anti-VEGF och anti-VEGFR-specifika antikroppar till friska möss för att utvärdera VEGFs roll vid stabiliseringen av kärlfunktionen i olika vävnader och organ.

Slutligen, utvärderade vi om expressionen av VEGF regleras av dygnsrytmen genom så kallade klock-gener. I paper I utvecklade vi en modell för hypoxi-inducerad retinopati hos vuxna zebrafiskar. Vuxna *flil:EGFP* zebrafiskar placerades i syrefattigt vatten i 3-10 dagar, varpå retinal nybildning av kärl analyserades. Denna modell ger en unik icke-invasiv möjlighet att studera kinetiskt utveckling av retinopati och den möjliggör bedömning av terapeutiska effekter av oralt givna anti-angiogena läkemedel. I paper II utvecklade vi en zebrafiskmodell för utvärdering av cancermetastaser, som möjliggör studier av detaljerade delprocesser vid hypoxi-inducerad tumörcellsinvasion och metastasering i samband med angiogenes på encellig nivå. I denna modell användes fluorescerande Dil-märkta humana- eller mustumörceller som implanterades vid den perivitellina håligheten hos 48-h-gamla zebrafiskembryon placerade i syrefattigt vatten i 3 dagar. Tumörcellsinvasion, metastasering och patologisk angiogenes analyserades med mikroskopi i levande fiskar. Vårt protokoll möjliggör studier av molekylära mekanismer bakom hypoxi-inducerad cancermetastasering. I paper III visas, att systemisk administration av anti-VEGF eller anti-VEGF-receptor (VEGFR)-2 neutraliserande antikroppar i en musmodell orsakar generell kärlregression. Bland alla undersökta vävnader påverkades endokrina körtlar, tarmslemhinna och uterus mest av VEGF eller VEGFR-2 blockad. Långvarig anti-VEGF behandling resulterade i en signifikant minskning av cirkulerande nivåer av det dominerande sköldkörtelhormonet, fritt tyroxin, men inte av trijodtyronin, vilket tyder på att kronisk anti-VEGF behandling försämrar sköldkörtelfunktionerna. Resultaten påvisar risken för biverkningar i friska vävnader av anti-VEGF behandling. I paper IV visar vi att störningar i dygnsrytm genom konstant exponering för ljus och genetisk manipulation av nyckelgener i zebrafisk ledde till nedsatt angiogenes under embryonal utveckling. En *bmal1*-specifik morfolino hämmade angiogenes i zebrafisk utan att orsaka andra kärl-oberoende fenotyper. Omvänt, en *Period2* morfolino accelererade angiogenes tillväxt, vilket tyder på att *Bmal1* och *Period2* utövar motsatta effekter på kärlstillväxt. Dessa resultat ger mekanistisk kunskap om den roll som dygnsrytmen har i regleringen av angiogenes, och resultat kan rimligen utvidgas till andra typer av fysiologisk eller patologisk angiogenes. Sammanfattningsvis ger resultaten i denna avhandling ytterligare kunskap om angiogenetiska mekanismer och pekar på möjliga nya terapeutiska mål för behandling av olika angiogenes-beroende sjukdomar.

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*Equal Contribution

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ABBREVIATIONS

AMD	Age-related macular degeneration
Bmal1	Brain and muscle ARNT-like-1
CBF-1	C-promoter binding factor 1
ChIP	Chromatin Immunoprecipitation
Clock	Circadian locomotor output cycles kaput
CNV	Choroidal neovascularization
Cry	Cryptochrome
CSL	CBF-1, Su(H), Lag-1
cyc	Cycl (gene)
DD	Constant darkness
Dll	Delta like ligand
DR	Diabetic retinopathy
ECM	Extracellular matrix
EGF	Epidermal growth factor
EPC	Endothelial progenitor cell
EMA	European medicines agency
FDA	Food and drug administration
FGF	Fibroblast growth factor
GI	Gastrointestinal
HBGF	Heparin binding growth factor
Hes	Hairy enhancer of split
Hey	Hes-related protein
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
hpf	Hours postfertilization
HRE	Hypoxia response element
Ig	Immunoglobulin
IL-6	Interleukin-6

IP	Intraperitoneally
ISV	Intersegmental vessel
KDR	Kinase inserted domain-containing receptor
Lag-1	Lin-12 and glp-1
LD	Switching between 12 h light and 12 h dark
LL	Constant light
MMP	Matrix metallo-proteinase
NEC	Notch extracellular domain
NICD	Notch intracellular domain
NVG	Neovascular glaucoma
OM	Optical microscope
PC	Pericyte
PDGF	Platelet-derived growth factor
Per	Period
PF-4	Platelet factor-4
PIGF	Placenta growth factor
qPCR	Quantitative real time-PCR
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription-PCR
SIV	Subintestinal veins
SMC	Smooth muscle cell
Su(H)	Suppressor of hairless
sVEGFR	Soluble VEGFR
TGF	Transforming growth factor
TKI	Tyrosine kinase inhibitor
T3	Free triiodothyronine
T4	Free thyroxine
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VPF	Vascular permeability factor
VPR	VEGF-related protein

INTRODUCTION

1 ANGIOGENESIS

1.1 Biological Functions of Vessels

During the embryonic development process in humans, the vasculature is the first developed functional system. Its main function is to deliver nutrients, oxygen and hormones secreted by the endocrine organs to tissues in the whole body, and to transport carbon dioxide, urea and other metabolites from the tissues and organs to eliminate from the body. It is well-established that the vasculature is an indispensable organ in maintaining normal metabolism and other physiological activities in the body. The vascular system includes cardiovascular system and lymphatic vascular system, with blood vessels sub-divided into arteries, veins and capillaries based on their structure and function.

1.2 Concept of Newly Formed Blood Vessels

The growth process of the vascular system is categorized into vasculogenesis, arteriogenesis and angiogenesis^{1,2}. In the embryonic vascular development stage and after birth, bone marrow-derived endothelial progenitor cells (EPCs) migrate to specific sites, where they differentiate into mature endothelial cells, and gradually form the vascular lumen and, eventually, the vascular network. This process is called vasculogenesis³. Arteriogenesis is the process in which blood flow is redirected into the collateral arteriole after the main artery is blocked, which causes cell proliferation and vascular remodeling, thereby forming enlarged functional arteries from smaller vessels^{4,5}. Angiogenesis refers to the neovascularization process from pre-existing vascular endothelial cells in the capillaries through continuous proliferation and migration^{4,6-10}.

1.3 Background of Angiogenesis

Dr. John Hunter first proposed the concept of “angiogenesis” in 1787 to describe the growth of new vessels during development in antlers of the Fallow Deer⁶. However, over the following hundred years, angiogenesis is rarely mentioned in

exploration of related diseases, and almost all focus are on anatomical studies. This was the case until the mid-19th century when Thiersch demonstrated that new vessels originated from preexisting capillaries¹¹. Later, it was also found that tumor tissues, relative to normal tissues, were rich in blood vessels, which was generally thought to be associated with inflammation. The relationship between the growth of blood vessels and the growth of tumors was not considered.

It was recognized early on that cells in humans and other mammals cannot live without blood vessels. Moreover, the responsibility of blood vessels for transportation and exchange of nutrients, gases, hormones and cells among the tissues and organs was given great importance. That blood vessels, or specifically the endothelium, themselves produce various active substances, which makes it a vital endocrine organ in the body was discovered in 1960s¹². Other important discoveries includes the findings that the distance limit between the cells and the vessels of 100-200 μ m in diameter is crucial to ensure diffusion of the oxygen from the red blood cells in the blood vessels into the cells. Accordingly, early developing primary solid tumors during the dormant period can maintain their growth only through penetration of the peripheral interstitial fluid^{11,13,14}. In 1971, Dr. Judah Folkman proposed for the first time that the growth and metastasis of solid tumors were dependent on angiogenesis⁸.

1.4 The Angiogenic Switch

Under normal conditions, angiogenic activators that can stimulate angiogenesis and angiogenic inhibitors that prevent angiogenic processes are responsible for regulating angiogenesis. The activation of angiogenesis is called the “angiogenic switch”. When activators of angiogenesis become dominant, the angiogenic switch is turned on. Angiogenic activators includes growth factors such as vascular endothelial growth factor (VEGF)¹⁵, platelet-derived growth factor (PDGF)¹⁶, fibroblast growth factor (FGF)^{17,18}, and transforming growth factor (TGF). Angiogenesis inhibitors include angiostatin, thrombospondin-1, platelet factor-4 (PF-4), matrix metalloproteinase (MMP) inhibitors. These factors act directly on vascular endothelial cells leading to proliferation and migration of vascular endothelial cells.

1.5 Process of Angiogenesis

Growth factors, in particular VEGF, promote angiogenesis. VEGF is a heparin binding growth factor that specifically acts upon endothelial cells. VEGF, among several angiogenic factors, can directly stimulate endothelial cells to make them migrate and proliferate. Vascular endothelial cells are a layer of tabular epithelial cells that form the inner walls of blood vessels. Under normal conditions, these cells display a long interval cycle of division and proliferation, and they are some of the most long-lived human cells. Under physiological and pathological angiogenesis, the activated cells can secrete proteases required for angiogenesis, and these proteases like MMPs are capable of degrading basement membrane and extracellular matrix to dissociate endothelial cells. Meanwhile, the endothelial cells are able to proliferate and migrate along the concentration gradient of growth factors (e.g., VEGF and FGF), whereby they become tip cells and stalk cells.

Tip cells lead the sprouting vessels, by extending filopodias along the gradient of angiogenic stimuli as a directional guidance, and induce bud growth^{19,20}. Adjacent stalk cells, that generate the trunks of the vessels, proliferate to form capillary-like lumens^{20,21}, and are covered by mural cells, pericytes (PCs) and vascular smooth muscle cells (SMCs). Ultimately, vascular networks are established and the vasculature becomes mature²².

In healthy humans, wound healing, the menstrual cycle and fetal growth and development are accompanied by physiological angiogenesis²³. Pathological angiogenesis takes place during tumor growth, rheumatoid arthritis, retinopathy, ischemic cardiocerebral vascular diseases, obesity, diabetes, and other various diseases²⁴⁻³⁰. A sufficient grasp of the knowledge about newly formed vessels is vital to the study how angiogenesis is implicated in all these diseases (see Figure 1).

1.6 ANGIOGENESIS IN DISEASES

Vascular homeostasis is an important foundation of the vital functions in the body and it plays an important role in maintaining the normal physiological state. The key for maintaining vascular homeostasis is a balanced and functioning vascular network. Normally, when the networks have been established, the endothelial cells remain in a quiescent state and no new vessels develop.

Figure 1. Angiogenesis in disease.

Nevertheless, under the influence of physical, chemical, biological and other internal and external environmental changes, the balanced system controlling blood vessel growth might be disrupted, and the development of new vessels is initiated, and the resulting process and final vessel network might even be chaotic. Thereby, angiogenesis occurs and the function and structure of the blood vessels change, which is a consequence or a prerequisite for pathological course of disease. Targeting angiogenesis, therefore, is a potential target for the treatment of cancer. Likewise there are ischemic diseases with a main feature of lack of delivery of oxygen and nutrients to tissue. The angiogenic process is then impaired and the balanced system is set at a very low level of new vessel formation in spite of the great need³¹. Then, promoting angiogenesis may be a beneficial therapeutic strategy.

1.6.1 Angiogenesis in Retinopathy

According to many studies pathological angiogenesis is closely related to VEGF expression, which for example may lead to development and occurrence of a variety of eye diseases³²⁻³⁶, including diabetic retinopathy (DR), age-related macular degeneration (AMD), neovascular glaucoma (NVG), and retinopathy of

prematurity. These diseases induced by abnormal angiogenesis are all related to over-expression of VEGF. The majority of new vessels formed in these diseases are not structurally completed, and the resulting hemorrhage, fibrosis and fluid secretions often lead to blindness³⁷.

DR, one of the most common and serious complications of diabetic microangiopathy has become the primary cause of blindness in many countries^{38,39}. The retinal neovascularization eventually results in retinal and vitreous hemorrhage leading to tractional detachment of retina and consequently blindness.

The primary lesion of AMD is choroidal neovascularization (CNV), in which VEGF plays an important role. During CNV, choroid blood circulation changes and anaerobic conditions³⁶ occur resulting in HIF-1 α (hypoxia inducible factor-1 α) and increased VEGF signaling⁴⁰. Therefore, targeting these pathways may be a potential way to control CNV formation.

NVG is an angiogenesis-induced eye disease that results in angiotectasis in the iris and anterior chamber. The accompanying intraocular hypertension often causes blindness as a result of this. Angiogenesis in the iris and anterior chamber angle is induced by ischemic conditions resulting in the release of VEGF due to hypoxia. Studies^{41,4241,4241,4241,4241,4241,4241,4240,4139,4038,3937,3837,3837,38} have indicated that VEGF levels in the aqueous fluid and vitreous body significantly increases in NVG-related eye diseases.

Although risk factors for many eye diseases not are clear, many drugs for treatment of angiogenesis-related eye diseases that are available that are based on the mechanism of inhibition of VEGF. Bevacizumab (with the trade name Avastin), an anti-VEGF antibody and the first FDA (U.S. Food and drug administration)-approved anti-angiogenic and anti-cancer agent in 2004, can inhibit proliferation of endothelial cells and reduce vascular permeability resulting in the blockage of neovascularization⁴³. With in-depth understanding of bevacizumab through a large number of clinical trials, it has been applied increasingly in many different diseases⁴⁴, also outside the eye. Additionally, it has demonstrated very significant clinical efficacy in the treatment of vascular proliferation-induced eye diseases⁴⁵⁻⁵¹. Results from clinical trials have certified

that intravitreal injection of bevacizumab is safe and effective treatment method, and that this agent can stabilize or improve the visual acuity, and effectively control the intraocular pressure of patients. Furthermore, few inflammatory or other complications have been recorded⁵².

In June 2006, the FDA approved ranibizumab (trade name Lucentis) for treatment of exudative AMD. It nonspecifically binds to activated VEGF thus blocking the interaction between VEGF and its receptor, thereby decreasing neovascularization⁵³. The European medicines agency (EMA) also approved ranibizumab for treatment of visual impairments induced by wet-AMD, diabetic macular edema, macular edema secondary to retinal vein occlusion and myopic choroidal neovascularization. In a recent phase III, double-blind, randomized, positively controlled clinical trial, it was shown that an intravitreal injection of ranibizumab was more effective than other therapies in treating myopic CNV⁵⁴.

1.6.2 Angiogenesis in Cancer

Currently, many diseases are known to be related to angiogenesis and cancer is the most widely studied of these diseases. Cancer, also known as neoplastic disease, is divided into malignant and non-malignant and it is one of the most common diseases in humans that are caused by uncontrolled cell growth and proliferation. Through many studies, the biological hallmarks of cancer have been revealed to include; persistent proliferation signals, insensitivity to growth inhibition, apoptosis resistance, immortalization, induction of angiogenesis, activating invasion and metastasis, reconstructing metabolic system of energy and avoiding destruction by the immune system⁵⁵. Methods to treat tumors have been studied for a long time. As part of these studies, angiogenesis mediated by tumor-derived growth factors has become one of the most important targets for tumor therapy research.

Because endothelial cells in newly formed vessels proliferate rapidly, they are very sensitive to chemotherapeutic drugs. However, specific agents that target the angiogenic process have also started to be developed. So far, anti-angiogenic therapy is recognized as very promising anti-cancer therapy strategy. Work in this field stems from Dr. Judah Folkman's theory, postulated more than 40 years ago, where he proposed that the growth and metastasis of all solid tumors was

dependent on blood vessel growth, and thus inhibition of angiogenesis could be an effective means of cancer treatment⁸.

Tumors do not generally grow beyond 2mm³ without oxygen and nutrients provided by blood vessels. Therefore, angiogenesis is the key factor to support continuing tumor growth. Growth factors secreted by the tumor cells promote growth and development of new blood vessels. Tumor neovascularization, however, is completely different from vessels in the host tissue. Morphologically tumor vessels are irregular, torturous and heterogeneous, leaky and with an abnormal basement membrane⁵⁶. Therefore studies of angiogenesis and the mechanism behind it are fundamental and crucial for treatment of cancer.

1.6.3 Angiogenesis in Cardiovascular Disease

Research of the mechanism of neovascularization can not only lead to the possibility of inhibiting the growth of blood vessels to treat different diseases, but also suggest therapeutic targets for ischemia-related diseases, such as coronary heart disease, peripheral arterial disease and cerebral thrombosis.

The reduction of perfusion or inhibition in bloods ability to deliver and remove molecules to organs or tissues results in ischemia. Ischemia is generally caused by local hemodynamic disorders in larger vessels, and sometimes by local manifestations of this anematososis. At present, ischemic heart disease is a leading disease worldwide in causing morbidity and mortality. Coronary hypoperfusion-induced myocardial ischemia and the resulting hypoxia in the heart muscle is the main problem in advanced ischemic heart disease causing both myocardial infarction and long term also congestive heart failure. Strategies, that may be able to complement interventional treatment for such diseases, can involve the infusion of proangiogenic factors into the myocardium or ischemic area resulting in stimulation of the formation and maturation of new vessels to enhance blood perfusion to the ischemic area. Animal experiments have demonstrated that proangiogenic factors, such as VEGF, can be administered to the ischemic myocardium using a carrier protein or gene via cardiac catheterization and this minimally invasive treatment method may cause increased angiogenesis⁵⁷⁻⁶⁰. Phase I clinical trials using infused growth factors has been conducted in patients with coronary heart disease^{61,62}. In one of these trials, growth factors were injected into the ischemic area of myocardium, and it was observed that the score

assessing the function of collateral vessels increased. More importantly, the results also suggested that proangiogenic gene therapy through intramyocardial injection was safe and tolerable. In the latter study it was impossible to objectively assess how efficacious the experimental treatment due to lack of control group. Further studies and clinical trials are needed to confirm the effectiveness and safety of proangiogenic therapy in cardiovascular diseases. In addition, VEGF not only promote neovascularization of ischemic myocardium, but also enhance blood perfusion of normal myocardium without pathological alterations. Therefore, injection of VEGF into non-ischemic myocardium may even be an effective prevention strategy for early ischemic heart disease⁶³.

1.6.4 Circadian Rhythm and Related Diseases

The circadian clock is a widespread vital phenomenon influencing living things, including prokaryotic and single-celled organisms, other mammals and humans. The basis for the circadian clock is the expression of circadian clock genes. The day-night rhythm, the most common circadian rhythm in nature, refers to an adaptive response of living beings to the light-dark cycle in their environment, allowing the body behavior and physiological activity to act continuously throughout a 24-hour cycle^{64,65}. Many physiological behaviors are regulated by day-night rhythms in human and other mammals including feeding/fasting and activity/resting^{66,67}.

The genes involved in circadian rhythms in organisms include circadian locomotor output cycles kaput (*Clock*), brain and muscle ARNT-like-1 (*Bmal1*), period (*Per*), cryptochrome (*Cry*), cycle (*Cyc*) amongst others⁶⁸⁻⁷¹, and these genes participate in many physiological and biochemical activities in the body, including cell growth and differentiation⁷². A series of physiological processes *in vivo* are closely associated with regulation of the expression of circadian clock genes, for instance blood pressure regulation, respiration rate, heart rate, metabolism and hormone secretion^{66,73-76}. Studies have confirmed that disturbed day-night/light-dark rhythm might induce many diseases including cancer, diabetes, myocardial infarction, obesity, stroke, depressive disorder and rheumatoid arthritis⁷⁷⁻⁸³. Additionally, these diseases are often accompanied by pathological changes in vascular structures and vascular function^{27,84-87}, including the occurrence of angiogenesis.

Circadian clock genes (e.g., *Bmal1*) may regulate angiogenesis by the influence of the circadian rhythm. As such, VEGF expression levels change with the circadian rhythm. Investigating circadian clock genes in relation with angiogenesis, therefore, would enable better understanding of the mechanism of pathological angiogenesis in diseases caused by disrupted circadian rhythm.

2 VEGF FAMILY OF PROTEINS

2.1 Background of VEGF

As mentioned before is the vasculature pivotal in development, maturation and homeostatic maintenance of vertebrates. The establishment of vessels depend on growth of endothelial cells that is stimulated by various growth factors (e.g., VEGF, PDGF, FGF, epidermal growth factor (EGF), TGF, etc.)⁸⁸. Growth factors are polypeptides that can bind to specific receptors on the plasma membrane to switch on a fast protein cascade which leads to DNA replication and cell division.

These growth factors activate quiescent endothelial cells leading to the process of physiological or pathological neovascularization. Among them, the VEGF family of multifunctional cytokines directly or indirectly regulates vasculogenesis, angiogenesis and lymphangiogenesis.

In 1983, Senger et al identified a factor that efficiently increased the vascular permeability in animal tumor models, and they called it vascular permeability factor (VPF)⁸⁹. In 1989, Ferrara and his colleagues isolated and purified a factor that was widely distributed in the tissues of human and mammals that specifically could promote vascular endothelial cell mitosis, which they named VEGF⁹⁰. In the subsequent studies VPF and VEGF were confirmed to be the same protein encoded by the same gene consisting of two single-strands. VEGF was shown to have a lower synthesis levels in healthy tissues of adult humans and animals, but higher expression in the embryo and during physiological or pathological neovascularization.

VEGF has an extremely strong ability to induce vascular permeability⁸⁹. Generally, increased vascular permeability is necessary for physiological

processes, but it can often lead to negative consequences during pathological processes. For example, increased vascular permeability in a tumor might aid metastasis formation from a primary tumor.

The members of the VEGF family are dimeric glycoproteins with a molecular weight of between 34 to 45kDa. This family can be divided into five isoforms; VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PlGF) in humans and other mammals. In addition, a protein related to the structure of VEGF was noted in the Orf virus, which came to be known as VEGF-E⁹¹. Another group of proteins called VEGF-Fs were found in snake venom isolated from the Horned viper and Russell's viper^{92,93}. Each isoform of VEGF forms dimers and bind to their receptor causing dimerization of the receptor which consists of any combination of three VEGF receptors (VEGFRs) as shown in Figure 2⁹⁴. Although there are various combinations for each of the ligands and receptors, each combination would promote formation and development of vessels, lymph vessels and other vasculatures.

Figure 2. The VEGF family and VEGF receptors.

2.2 VEGF ligands and Functions

2.2.1 VEGF-A

VEGF-A, usually called VEGF, is a highly specific vascular endothelial cell mitogen. Furthermore, it is also the strongest pro-angiogenic factor in VEGF family and it is expressed in every organ and tissue in the body^{95,96}. Hence, it is one of the most studied growth factors. The VEGF-A gene, containing eight exons and seven introns in the coding region, generates various isoforms after transcription, mRNA splicing variation and other steps that all have different biological properties. Among them, the isoforms of human VEGF-A are labeled as VEGF-A121, VEGF-A145, VEGF-A165, VEGF-A189, VEGF-A206^{97,98}. The functional difference among these isoforms is reflected by different binding activity between them and the heparin in the extracellular matrix. VEGF-A165 is the most common isoform that shows a major effect^{99,100}.

2.2.2 VEGF-B

VEGF-B has a relatively high expression in myocardium and skeletal muscle¹⁰¹. VEGF-B has two different transcripts, VEGF-B167 and VEGF-B186. VEGF-B186 is secreted from the cytoplasm into the extracellular matrix, while VEGF-B167 is secreted only under the stimulation by heparin. Furthermore, VEGF-B binds to VEGF-A to form a heterodimer.

2.2.3 VEGF-C

VEGF-C with the molecular weight of 46.9kDa was purified first by Joukov et al. in 1996¹⁰². Lee et al. also isolated the corresponding gene, and the protein encoded by this gene was named VEGF-related protein (VRP)¹⁰³. VEGF-C is a mitogen for lymphatic endothelial cells, and is involved in the development of lymphatic system^{104,105}, and can promote lymphangiogenesis and induce lymphatic hyperplasia^{106,107}. Its role in mediating lymphangiogenesis is caused by its ability to effectively regulate activation, proliferation and migration of lymphatic endothelial cells. As lymph node metastasis may occur by invasion of tumor cells into the lymphatic vessels, VEGF-C, is closely associated with the tumor metastasis¹⁰². Clinical data has demonstrated a correlation between VEGF-C levels in the tumor and metastasis process^{108,109}. Additionally, VEGF-C may induce microvascular endothelial cell proliferation to some extent due to its ability to bind to VEGFR-2¹¹⁰.

2.2.4 VEGF-D

VEGF-D is similar to VEGF-C in structure. VEGF-D mRNA can be detected in the majority of human tissues, and is relatively abundant in the myocardium, lung, skeletal muscle, colon, and small intestine of adults¹¹¹. It not only induces lymphangiogenesis in tumors, but also causes diffusion of the tumor cells towards the regional lymph nodes through its expression in the tumor cells¹¹².

2.2.5 VEGF-E

VEGF-E with the molecular weight of 20kDa is absent in humans and other mammals. It is, however, found to have the similar biological effects as VEGF-A165. At the protein level it demonstrates a ~25% sequence homology with VEGF-A165 at the amino acid level, though it lacks the basic domain and heparin binding domain¹¹³.

2.2.6 PlGF

PlGF is a secreted glycoprotein, and is a dimer that first was isolated and purified from cDNA in human placenta by Iyer et al¹¹⁴. There are four family members in this group, PlGF-1, PlGF-2, PlGF-3 and PlGF-4 based on the different mRNA splicing variants. PlGF is only be expressed in various kinds of tumors, but also in placenta in large amounts^{115,116}, which lasts the entire pregnancy, hence its name. PlGF can also bind to VEGF-A to form a heterodimer¹¹⁷.

2.3 VEGF Receptor and Signaling

The activation of the classical VEGF biological signal transduction pathways require the specific binding of VEGF ligands and three types of transmembrane receptor tyrosine kinases (RTKs) in the cell membrane (VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4)) as. Among them VEGFR-2 is expressed in all endothelial cells and is the most important receptor mediating vasculogenesis. Human VEGFR-2 is called kinase inserted domain-containing receptor (KDR), while the murine receptor is called fetal liver kinase-1 (Flk-1).

2.3.1 Structure of VEGF Receptors

VEGFRs are members of the RTK superfamily and made up of one extracellular domain, one transmembrane domain and one intracellular domain^{118,119}. Among them, the extracellular domain is composed of seven immunoglobulin (Ig)-like folds⁹¹. The intracellular domain contains two tyrosine kinase domains with a C-

terminal tail at the end, and various cell signals are mediated from intracellular domain to downstream of signaling cascades¹²⁰.

The activation of VEGFRs is controlled by its ligand. After the extracellular VEGFs dimer binds to the corresponding VEGFRs, the monomeric vEGFRs dimerize. According to the differences of VEGFs dimer structures and the specific binding capacity to VEGFRs, the dimerized VEGFRs will form into a homodimer or a heterodimer¹²¹. Dimerized tyrosine kinases are activated and induce phosphorylation of its own tyrosine residues resulting in the phosphorylation and activation of other downstream proteins and pathways including the activation of a series of the second messengers.

2.3.2 VEGF Receptor-1

Though VEGFR-1 was the first discovered RTK receptor of VEGF, its function is controversial due to its many roles. It regulates different signal pathways in various cell types and stages of the cell cycle. VEGF-A, VEGF-B and PlGF are all ligands of VEGFR-1. Due to various splicing variants, VEGFR-1 may also produce soluble VEGFR-1 (sVEGFR-1). VEGFR-1 and soluble VEGFR-1 can both bind to VEGF-A, with high binding affinity but low response level compared to VEGFR-2^{122,123}. Park et al. thought that VEGFR-1 is a decoy receptor that creates no active signal transduction for cell mitosis working as negative regulator of VEGF¹²⁴. The other possibility is that VEGFR-1 could block angiogenic cell signals through competing with VEGFR-2 for binding of VEGF-A. It may then lead to less VEGF binding to VEGFR-2. VEGF-B and PlGF can only bind to VEGFR-1 and not to VEGFR-2 and VEGFR-3. Ligand binded-VEGFR-1 induces phosphorylation in the intracellular domain resulting in the recruitment of VEGFR-1 expressing cells such as monocytes as well as secretion of some factors, such as Hepatocyte growth factor (HGF) and Interleukin-6 (IL-6).

2.3.3 VEGF Receptor-2

With its highly specific expression in endothelial cells¹²⁵, and as being the main functional receptor of VEGF-A, VEGFR-2 plays a leading role in VEGF mediated signaling and vascular endothelial growth. VEGF-A bound to VEGFR-2 activates a series of intracellular downstream signal molecules related to mitogenic, chemotactic and anti-apoptotic effects. Under stimulation of VEGF-A,

tyrosine residues at different locations on VEGFR-2 can bind to multiple proteins, which mediate signals and induce phosphorylation and initiation of a signal cascade reaction, regulating the biological characteristics of endothelial cells through different activation pathways (e.g., endothelial cell migration, cell proliferation, actin remodeling and anti-apoptosis). Eventually, VEGF-A-VEGFR-2 signaling promotes angiogenesis. Coupled with this effect on angiogenesis, VEGFR-2 can also participate in VEGF-A-mediated increases in vascular permeability¹²⁶⁻¹²⁸.

VEGF-C and VEGF-D also bind to VEGFR-2. Even though VEGF-E is absent in humans and other mammals, VEGF-E can bind specifically to VEGFR-2 and mediate the biological effects similar to VEGF-A. For example, in the rat tumor and cornea models, angiogenesis mediated by VEGF-E can be detected and new vessels are similar to those produced by VEGF-A mediated signaling in their form, structure and function.

2.3.4 VEGF Receptor-3

VEGFR-3 is mainly expressed on endothelial cells of lymphatic vessels^{104,129} and is the specific receptor of VEGF-C and VEGF-D. VEGF-C and VEGF-D bound VEGFR-3 activate downstream signaling pathways and induce lymphatic endothelial cell proliferation, migration and formation of the lymphoid sinus¹³⁰, thus causing lymphatic hyperplasia. VEGFR-3 also plays a vital role in cardiovascular formation during embryonic development. During late embryonic development VEGFR-3 is expressed mainly in lymphatic vessels and to a small degree in blood vessels. Only one layer of endothelial cells with an irregular basement membrane forms the lymphatic capillaries^{131,132}. Malignant tumor cells can therefore enter the lymphatic vessels easily, whereas tumor cells need to penetrate a well-aligned basement membrane to intravasate blood vessels¹³³.

3 CELL SIGNALING IN ANGIOGENESIS

3.1 Hypoxia in Angiogenesis

Pathological angiogenesis is often accompanied by tissue hypoxia¹³⁴. Hypoxia causes a series of alterations of processes in tissues and cells to allow adaptation to low oxygen environments. For example, under hypoxic conditions angiogenesis, cell proliferation, cell survival, ion metabolism and carbohydrate

metabolism change, and genes that are involved in the hypoxic response are induced¹³⁵. Among them, hypoxia-inducible factor-1 (HIF-1) is a vital transcriptional regulatory factor in such adaptations¹³⁶. HIF-1 transcribes and activates the expression of a series of genes under hypoxia, and especially upregulation of VEGF¹³⁷. Since hypoxia can induce functional alterations in the body, it is recognized as one of the most prominent processes that cause diseases even leading to death. This has led to a great number of studies evaluating angiogenesis under hypoxic conditions.

3.2 HIF/VEGF signaling pathway

3.2.1 Structure and Functions of HIF

HIF is a transcription factor that is upregulated when tissue ischemia and hypoxia cause a lowered oxygen concentration in the cells. HIF induces certain regulatory effects. The HIF family includes HIF-1, HIF-2 and HIF-3¹³⁸. Among them, HIF-1 is a heterodimer transcription factor comprised of protein subunits α and β . They were first discovered in hypoxia-induced cell extracts by Semenza et al. in 1992¹³⁹. HIF-1 α is the main regulator of the hypoxic response and is highly sensitive to the oxygen concentration. It only consists of an oxygen regulation subunit^{140,141} therefore it determines the activity of HIF-1 depending on oxygen concentration¹⁴². HIF-1 β shows constitutive expression in cells and is not affected by the oxygen concentration^{143,144}. When HIF-1 α forms a dimer with HIF-1 β , HIF-1 is activated.

3.2.2 HIF Pathway

Under normoxic conditions, HIF-1 α is degraded by proteasomal degradation¹⁴⁵. Under hypoxic conditions owing to hydroxylation reaction inhibition, it cannot be degraded, leading to accumulation in cells. Then HIF-1 α enters into the nucleus and binds to HIF-1 β to form heterodimers. Following that process, the heterodimer binds specifically to the hypoxia response element (HRE) on the VEGF promoter or enhancer. This increases the stability of VEGF mRNA and also promotes up-regulation and transcription of VEGF and its receptors' expression. Hypoxia is the main stimulus of VEGF¹⁴⁶, which binds specifically to VEGFR on vascular endothelial cells to activate a series of transduction pathways in response to ischemia to induce neovascularization¹⁴⁷.

3.3 Notch signaling pathway

3.3.1 Background of Notch

The Notch signaling pathway plays different roles in various processes during development. This signaling is essential in ensuring normal embryonic development and maintaining steady state of tissues and stem cells¹⁴⁸. In 1919, the Notch gene was first discovered in fruit flies and it is an evolutionarily highly conserved family of transmembrane receptors¹⁴⁹. The Notch signaling pathway comprises of Notch ligand, Notch receptor and CSL (C-promoter binding factor 1 (CBF-1), suppressor of hairless (Su(H)), lin-12 and glp-1 (Lag-1)) DNA-binding protein¹⁵⁰. Upon binding of the Notch ligand to its receptor, Notch signal transduction is activated and this regulates cell differentiation and histogenesis. Mutation of Notch or its ligand may lead to developmental defects in the heart, skeleton, hematopoiesis- and nervous system. It is also shown that Notch is of significance in various processes of vasculogenesis and angiogenesis^{151,152}.

Structure and Functions of Notch

There are four different Notch receptors in vertebrates, i.e., Notch1, Notch2, Notch3 and Notch4. Structurally the Notch receptor consists of Notch extracellular domain (NEC), a transmembrane domain and the Notch intracellular domain (NICD). The main function of the NEC is to bind to ligands on adjacent cells to activate the Notch signaling pathway. The Notch ligand is a single-pass transmembrane protein expressed on the cell surface and there are five types in mammals: jagged1, jagged2, Delta like ligand 1 (Dll1), Delta like ligand 2 (Dll3) and Delta like ligand 4 (Dll4). Notch signal transduction requires several steps during the activation process. In detail, after the Notch ligand bind to the receptor, a site on the membrane of Notch receptor is cut by γ -secretase to release NICD. Then, NICD enters the cytoplasm and is transferred into the cell nucleus. Finally, NICD interacts with CSL and converts CSL to a transcriptional activator^{153,154}, the downstream transcriptional factors, such as hairy enhancer of split (Hes) and Hes-related protein (Hey) families, are targets of Notch/CSL-dependent signaling¹⁵⁵⁻¹⁵⁷ and further regulate the expression level of downstream molecules to regulate expression of cell differentiation-related genes^{158,159}.

3.3.2 Dll4: The Ligand of Notch

Dll4 is one of the ligands of the Notch signal family in mammals¹⁶⁰. Dll4, much like VEGF, is the gene related to vascular growth and development. It is

expressed during physiological and pathogenical angiogenesis and plays a key role in vasculogenesis and angiogenesis as well as in maintaining vascular homeostasis¹⁶¹. Dll4, therefore, has potential clinical applications^{162,163}. In particular Dll4, which is not only expressed restrictively in the aortic endothelial cells during embryonic development^{151,160,164}, but also the only ligand expressed in tip cells during vascular budding¹⁶⁵. The Dll4/Notch signal plays a key role in the process of differentiation from tip cell to stalk cells in endothelial cells.

Dll4 belongs to a group of hypoxia regulatory genes¹⁶⁶. In the classical HIF-1 α signaling pathway, after Dll4 ligands and receptor binding, NICD and HIF-1 α interact with each other and during hypoxia HIF-1 α is recruited as a Notch response promoter to activate Notch signaling. Therefore hypoxia promotes and stabilizes the activity of NICD¹⁶⁷, and strengthens the final signal expression. Many details, however, of the Notch signaling pathway and the connections between different cell signaling pathways are still undefined.

Experimental studies have demonstrated that VEGF can induce the tip cell phenotype¹⁶⁸, and the expression of Dll4 in endothelial cells regulates the budding and branching of new vessels. Further research confirmed that angiogenesis is under the control of VEGF and Dll4/Notch signaling pathways' synergistic effect. VEGF interacts with Dll4/Notch signaling pathway, that is, on one hand, VEGF induces Notch signal, on the other hand, Notch signal can act reversely on VEGF, regulating the strength of the VEGF signal pathway to allow angiogenesis to proceed in an orderly way¹⁶⁹.

Vasculogenesis is influenced by, and strictly regulated by endogenic vessel growth regulator. There are two types of vasculogenesis regulators: angiogenic stimulators and angiogenic inhibitors. The balance between these two types of factors and their biological functions influence the results of physiological or pathological angiogenesis.

4 ANGIOGENIC EFFECTORS

Angiogenesis is the result of co-regulation of VEGF and many other growth factors and cytokines¹⁷⁰. For instance, PDGF is an important mitogenic factor,

and is a multi-functional protein synthesized and excreted by platelets, endothelial cells, vascular smooth muscle cells and fibroblasts amongst others, and is able to stimulate the division and proliferation of various cell types.

4.1 Other angiogenic stimulators

4.1.1 PDGF Family

PDGFs is a dimer composed of two highly isogenous chains of -A, -B, -C, or -D through disulfide bonds. There are five isoforms: PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD, and the heterodimer PDGF-AB¹⁷¹. PDGFs must bind to the corresponding PDGFRs on the cell membrane in order to have a biological effect. When PDGFs bind to PDGFRs, dimerization occurs and produces three receptor isoforms, PDGFR- $\alpha\alpha$, PDGFR- $\beta\beta$ or PDGFR- $\alpha\beta$. PDGF-AA binds only to PDGFR- $\alpha\alpha$, while PDGF-CC binds to both PDGFR- $\alpha\alpha$ and PDGFR- $\alpha\beta$. PDGF-DD binds only to PDGFR- $\beta\beta$, while PDGF-BB binds to all three receptor isoforms, PDGFR- $\alpha\alpha$, PDGFR- $\beta\beta$ and PDGFR- $\alpha\beta$. There are evidence that PDGFs and the corresponding receptors play a role in regulation of microvascular endothelial cell proliferation and migration, which results in neovascularization^{172,173}.

PDGF is not only a strong mitogen, but also interacts with other factors. For example, it promotes fibrosis together with TGF- β ¹⁷⁴, and induces active expression of MMPs. MMPs are a set of enzymes that are capable of degrading almost all kinds of protein components of the extracellular matrix (ECM). Therefore, it plays an important role in the process of tumor invasion and metastasis, as well as in angiogenesis in general.

4.1.2 FGF Family

FGF is a superfamily of proteins that is responsible for a variety of functions in metabolism, maintenance of organization structure during development as well as hemostasis. It has its effects through paracrine and autocrine signaling. Due to its high affinity to heparin, it is also known as the heparin binding growth factor (HBGF)¹⁷⁵. FGFs are found in both vertebrates and invertebrates. So far, at least 23 kinds of FGF proteins have been identified in humans and rats. It is divided into FGF-1 (FGF acidic) and FGF-2 (FGF basic) according to differences in structure, but both have effects in various biological processes such as

angiogenesis, wound healing, cell proliferation, differentiation and cell survival¹⁷⁶.

FGFs' activity is mediated through four types of FGFRs, all with different coding genes. These four highly conserved RTKs (FGFR-1, FGFR-2, FGFR-3 and FGFR-4) are similar to most growth factor receptors¹⁷⁷. FGFRs, a kind of transmembrane protein, contain an extracellular domain, a transmembrane domain and an intracellular domain, which provide the molecular basis of receptors for the binding of ligands and signal transduction¹⁷⁸. Additionally, there is a decoy receptor, FGFR-5, which also binds to FGF ligands, but has no effect on proliferation¹⁷⁹.

Dimerization occurs when FGFRs bind to ligands, and phosphorylated FGFR tyrosine kinases further activate downstream pathways. Many experiments have shown that FGF and VEGF signaling, or other growth factor signal pathways such as PDGF have a number of important functions in angiogenesis, lymphangiogenesis and tumor metastasis¹⁸⁰⁻¹⁸³.

4.2 Angiogenic Inhibitors

Angiogenic inhibitors generate anti-angiogenic activity by affecting many different parts of the angiogenetic process, for example, ECM reconstruction, endothelial cell migration and proliferation, and microvascular lumen formation. Endogenous inhibitors of angiogenesis could be divided into two groups based on their different specifics. One type of angiogenic inhibitor act specifically on endothelial cells, and the other has effects on other cell types as well as endothelial cells¹⁸⁴⁻¹⁸⁶.

4.2.1 Angiostatin

In 1994 Angiostatin was first isolated from the serum and urine of a murine Lewis lung carcinoma model and was found to have a molecular weight of 38kDa. Angiostatin acts specifically on endothelial cells, and inhibits their proliferation and migrations and induces apoptosis by binding to receptors on the surface of endothelial cells^{187,188}.

Animal experiments have indicated that the therapeutic effect on tumors of angiostatin is stronger than common cytotoxic therapy. Its advantage is that angiostatin is appropriate for long-term usage because it does not display the

acquired drug-resistance that is common. Currently, angiostatin has entered clinical trials in humans, and no dosage-limiting toxicity has been noted so far¹⁸⁶.

4.2.2 Endostatin

Endostatin is an angiogenic inhibitor with a molecular weight of 20kDa that first was isolated and purified from plasma of mice with hemangioendothelioma in 1997¹⁸⁹. Endostatin inhibits the migration of endothelial cells and influences survival¹⁹⁰. Endostatin can also induce expression of anti-apoptotic proteins in endothelial cells by significantly reducing the level of pro-apoptotic factors leading to increased cell survival¹⁹¹. Furthermore, studies showed that endostatin can modulate multiple signaling pathways of vasculogenesis, including VEGF and FGF-induced signal transduction pathways¹⁹².

Endostatin down-regulates numerous key regulators in the pro-vasculogenesis signaling pathways such as HIF-1- α gene transcription and reduces the expression levels of related factors in signaling pathways (i.e., VEGF and TNF- α) at the same time. In addition, it induces dephosphorylation of various proteins related to the vasculogenesis signaling pathways to inhibit their functions. Endostatin negatively regulates angiogenic factors while positively regulating angiogenic inhibitors. These two effects act synergistically to inhibit the vasculogenesis.

Endostatin was the first blood vessel growth inhibitor entering clinical trials due to its strong anti-tumor activity. The results of previous clinical trials demonstrated the safety of endostatin. The strong endogenous factors endostatin and angiostatin inhibit growth, proliferation and migration of endothelial cells, and consequently restrain the growth of tumor cells and make them to enter a dormant state^{187,189}.

4.3 Antiangiogenesis Compounds

Different from other traditional anti-tumor drugs, anti-angiogenic compounds target normal endothelial cells. They inhibit growth and metastasis of tumors. If tumors display drug-resistance to common cytotoxic therapy anti-angiogenic compounds may be the ideal drugs for treating cancer patients. FDA has approved several angiogenic inhibitors for tumor treatment in recent years, such

as bevacizumab, sunitinib malate and sorafenib. Additionally, several angiogenic inhibitors are still under development and in the near future they will be evaluated in clinical trials.

4.3.1 Bevacizumab

Bevacizumab (trade name Avastin) is a humanized monoclonal antibody targeting VEGF-A, was developed by Genentech. It was approved by FDA in February 2004 and was the first VEGF inhibitor that came onto the market. Bevacizumab can selectively neutralize VEGF-A, but does not interact with other VEGF family members such as VEGF-B, VEGF-C, VEGF-D or VEGF-E. Moreover, bevacizumab specifically inhibits the biological function of VEGF-A, and thus influence mitogenic activity of endothelial cells, vascular permeability and vasculogenesis activity¹⁹³. Preliminary clinical studies indicated that bevacizumab effectively could inhibit growth of several transplanted human tumor cells¹⁹⁴, but *in vitro* research failed to prove the inhibitory effect on human tumor cell lines. This indicated that the effect of bevacizumab was only related to neovascularization¹⁹⁵. The major adverse drug effects of bevacizumab include hypertension, rhinorrhea, fever, and occasionally coagulation and hemorrhage that can be life-threatening¹⁹⁶.

4.3.2 Sunitinib Malate

Sunitinib malate, (trade name Sutent), came into market in 2006¹⁹⁷. Being an oral multiple-target tyrosine kinase inhibitor drug, sunitinib can selectively target some protein receptors (e.g., VEGFRs and PDGFRs¹⁹⁸) that have a molecular switching function in the process of tumor growth. Sunitinib inhibits the growth and metastasis of tumor cells, significantly prolong the survival period and improve signs and symptoms cause by cancer. It can be applied widely and produces less adverse reactions than many other treatment options and is convenient for oral administration.

4.3.3 Sorafenib Tosylate

Sorafenib tosylate (trade name Nexavar) was approved by the FDA as an oral drug for treatment of late-stage renal carcinoma in December 2005. Sorafenib is a multiple kinase inhibitor with double anti-tumor effects. It inhibits numerous kinases including VEGFR-2, VEGFR soluble VEGFR-3 and PDGFR- β , and also

blocks the Ras-Raf-MAPK signal pathway to inhibit proliferation of tumors directly. Indirectly it suppresses tumor growth by preventing the neovascularization of the tumor.

4.3.4 VEGF-Trap

sVEGFR is one of the alternative splicing forms of VEGFR *in vivo*. sVEGFR only contains the extracellular Ig-like domain of VEGFR. The artificial recombinant sVEGF is called ‘Trap’ and was developed by Regeneron. The second Ig-like domain of VEGFR-1 and the third Ig-like domain of VEGFR-2 were linked to the Fc fragment of human IgG by means of genetic engineering^{196,199}. The recombinant protein has a longer half-life than the endogenous sVEGFR, and a higher binding affinity with VEGF¹⁹⁹. Also compared with bevacizumab VEGF-Trap has a higher binding affinity with VEGF, and it also binds to VEGF-B and PlGF. Experiments *in vitro* showed that the VEGF-Trap inhibits growth of various tumor cells. A low dose of VEGF-Trap produces similar efficacy levels as the humanized anti-VEGF monoclonal antibody. A high dose of VEGF-Trap not only inhibits growth of tumors, but also causes avascular tumors¹⁹⁹.

Ongoing development of anti-angiogenic or anti-tumor drugs targeting VEGF/VEGFR includes, for example, the humanized anti-VEGFR-2’s antibodies IMC-1C11, IMC-1121B²⁰⁰, and AG-013736²⁰¹.

5 ZEBRAFISH ANIMAL MODEL

The progress in medical and biological sciences is highly dependent of adequate animal models. Because embryology and genetics evolved through studies in *Drosophila*, it became one of the most important models for molecular auxology of invertebrates. However, the structure of embryos and the pattern of development between invertebrates and vertebrate are rather different. Therefore, research of embryogenesis, embryonic development, occurrence and development of diseases in human or vertebrates should be carried out in a vertebrate model. Mice and rats are often appropriate models but there is a limitation in their use for embryonic studies as their embryos develops in the uterus of the parent, making visualization difficult. Fish, on the other hand, are

gaining interest as a possible animal model for studying developmental biology due to a number of reproductive characteristics, such as their strong ability to reproduce, that spawning and fertilization can occur *in vitro*, and that the embryos are transparent. As a result, zebrafish are used as one of the most important representative vertebrates for research.

The first publication on zebrafish was published in 1981. In this publication the natural mutant "*golden*" was introduced in *Nature*²⁰², and by 1996, about 4000 mutations in zebrafish had been identified^{203,204}. Moreover, the sequencing of the zebrafish genome is almost completed. These rich genetic resources provide a solid basis for studies of molecular mechanisms in vertebrate growth. Due to available genetic manipulation techniques these circumstances gave the zebrafish model advantages. For example, *morpholino* oligos can be applied to knock down gene expressions, and delivery of mRNA can rescue this phenotype. Another advantage of using zebrafish embryos is their transparency and *ex vivo* development, which make them easy for microscopic analysis. Moreover, the diameter of the zygote is about 1mm, which allows for fine manipulation such as microinjection. Compared with mammals, zebrafish survives in a relatively hypoxic environment^{205,206}, which is favorable for studies to observe physiological or pathological blood vessel growth in a hypoxic environment.

More importantly, zebrafish embryos develop rapidly. Eggs complete the first mitosis in 40 minutes after fertilization at 28.5 °C, and then divide once every 15 minutes. The major basic organs begin to develop just one day post fertilization. Three months later, zebrafish embryos reach sexual maturity. Lastly, it is convenient to breed and culture zebrafish with a large number of groups in a limited space. Adult zebrafish lay eggs once a week, each time spawning between 100-300 eggs. This makes the zebrafish a cost-effective model.

Overall, by combining optimal animal models for angiogenesis research and of VEGF research in particular it is possible to gain further insight of the mechanism that is important in health and disease. Ultimately this may lead to improved cancer treatments as well as identification of new treatment targets for other diseases.

AIMS OF THIS THESIS

The overall aim of this thesis was to investigate the mechanism of VEGF-mediated vascular functions in health and disease.

Specific aims:

1. To develop optimal animal models able to clarify mechanisms of angiogenesis during hypoxic conditions.
2. To evaluate the effects and functions of VEGF signaling in healthy vasculature.
3. To reveal the role of the circadian clock in VEGF-induced angiogenesis.

METHODS

Zebrafish Model

This thesis contains three manuscripts that utilise an adult zebrafish model, as well as zebrafish embryos because of their many advantages over other animal models for our purposes.

Because of the advantages that were described in the introduction section of this thesis, we used a strain *Tg(fli1:EGFP)* zebrafish line in paper I and paper II as the main experimental animal model which expresses a green fluorescent protein in the blood vessel's ECs. All embryos were produced by natural mating, and maintained in standard 0.3× Danieau's or E3 embryos medium at 28.5 °C. Additionally, in paper IV, there are further studies using adult zebrafish as a model.

In paper I and II, use of an optical microscope (OM) enabled detachment of adult zebrafish retina and microinjections of tumor cells into the zebrafish embryos. A fluorescence microscope was used to perform qualitative and quantitative studies to identify the vasculature in *fli1:EGFP* zebrafish as well as tumor invasion and metastasis. The advantage is the ability to observe living cells dynamically using multiple fluorescent markers and track tumor dissemination using same fish at the different time points. Additionally, confocal microscope is also utilized in the experiments. Laser confocal scanning visualizes not only one layer of the image but also three-dimensional images, which provide detailed information of vessel structures and single tumor cells. This method is advantageous for studying angiogenesis.

Hypoxic Chamber

In order to create hypoxia environment for zebrafish in paper I and II, we designed a unique aquarium with a capacity of 1L. The hypoxic living environment was set up by adding nitrogen gas bubbles to water as very small bubbles and controlled by an oxygen regulator with oxygen concentration monitoring by an oxygen electrode.

Pharmacological Treatments and Morpholino Injection

For the study purposes, we applied drug treatments in different animal models, including oral medication, such as a γ -secretase inhibitor (DAPT) and ZN323881 added to the water environment of the zebrafish. Furthermore, morpholino, as a molecule for the modification of gene expression, was injected into zebrafish yolk at the single cell level. Morpholinos act by occupying the binding sites of the mRNAs to block the other molecules binding site to specific nucleic acid sequences.

PCR

In zebrafish experiments, RT-PCR (reverse transcription-PCR) and qPCR (quantitative real time-PCR) techniques was used. Zebrafish embryos were homogenized in buffer and RNAs were prepared using RNA purification kit. The expression levels of mRNAs detected are zebrafish *period2*, zebrafish *bmal1a*, zebrafish *vegfaa*, zebrafish *-actin*.

ChIP

Chromatin Immunoprecipitation (ChIP) is often used to study the interaction between proteins and DNA in cells. In this thesis' experiments, mouse fibroblast cells were used for the EZ-ChIP chromatin immunoprecipitation assay to determine binding of Bmal1 to the *vegf* promoter. A mouse *period1* fragment, known to bind to Bmal1, was used as a positive control. In brief, 100 μ l of sonicated chromatin was used for each immunoprecipitation reaction. A rabbit anti-Bmal1 antibody and a non-immune rabbit IgG were used for immunoprecipitation. The purified DNA was used for quantitative PCR analysis.

Mouse Model

Mice possess a high level of sequence homology in a number of genes with humans. Therefore mouse models were used in the experiments for studying systematic effects of anti-angiogenic drugs on whole organisms. In paper III we used 8-12 week-old female C57 black 6 (C57/Bl6) mice.

Mice were injected with a rabbit anti-mouse VEGF-specific neutralizing antibody (BD0801) and a rat specific antibodies to mice VEGFR-1 antibody

(MF1) and a rat specific antibodies to mice VEGFR-2 antibody (DC101) intraperitoneally (IP). The treatment schedules include:

- 1) Twice per week injection of the anti-VEGF antibody (5 mg/kg), anti-VEGFR-1 or anti-VEGFR-2 antibodies (200 µg per mouse) (n = 6 mice per group),
- 2) Twice per week injection of the anti-VEGF antibody (5 mg/kg) in the discontinuation experiment (n = 6 mice per group) for 2 weeks. Tissues were collected at day 0, 7, 14 and 28 after ending the anti-VEGF treatment (n = 6 mice per group), and
- 3) Twice per week injection of different dosage of the anti-VEGF antibody (1.25, 5, and 15 mg/kg) (n = 6 mice per group).

Histological Studies and Immunohistochemistry

In mouse experiments whole-mount staining and immunofluorescence techniques are used to visualize vessel structures in various organs. Collected tissue samples were fixed in 4% paraformaldehyde overnight and cut into thin slices followed by proteinase K (20 µg/mL) treatment. The sliced samples were incubated with a rat anti-mouse CD31 antibody (1:200) overnight at 4 °C, followed by staining with a goat anti-rat Alexa fluor 555 secondary antibody (1:200) for 2 h at room temperature. Slides were mounted using mounting medium (Vectashield) and vasculatures were visualized using a confocal laser microscope. Images were analyzed by Adobe Photoshop CS4 software. Paraffin-embedded tissues were sectioned at a thickness of 5 µm and stained with a rat anti-mouse endomucin antibody (1:200) followed by a goat anti-rat Alexa fluor 555 secondary antibody (1:400). The slides were mounted using Vectashield and images were photographed using fluorescence microscopy equipped with a camera. H&E staining were used for morphology studies. Samples were stained with hematoxylin followed by eosin after hydration of slides. Then slides were dehydrated and mounted. The images were obtained using light microscopy.

Western Blot

After 2 weeks anti-VEGF treatment, the bioreductive hypoxia marker pimonidazole (1.5 mg/mouse), was injected intravenously into the mice. They were then killed 15 minutes after the injection. The thyroid was collected and tissue lysate was used to assess levels of hypoxia. Proteins along with a protein ladder were subjected to SDS-PAGE followed by wet transferring onto

methanol-activated polyvinylidene fluoride membranes. Membranes were blocked with 5% skim milk solution for 1h before incubation with mouse anti-pimonidazole monoclonal antibodies overnight. Membranes were incubated for 45 min with a donkey anti-mouse IgG antibody was used. Positive signals were visualized using a chemiluminescence detection to identify the hypoxic indicated protein levels. Mouse β -actin was used as a loading control.

Vascular Perfusion Assay

At the end of vehicle- and anti-VEGF antibody treatment, mice were injected with a lysinated fluorescein-labeled dextran (2,000kDa, 1mg in 100 μ L per mouse) and killed 10 minutes after injection. The thyroids were resected and fixed in 4% (wt/vol) PFA at 4 °C overnight and cut into small pieces under a light microscope. After proteinase K (20 μ g/mL) treatment, sliced samples were incubated with a rat anti-mouse CD31 antibody (1:200) overnight at 4 °C, followed by staining with a goat anti-rat Cy5 secondary antibody (1:200) for 2 h at room temperature. Slides were mounted using mounting medium (Vectashield). Vasculatures and injected lysinated fluorescein-labeled dextran were visualized using a confocal laser microscope. The vessel area with dextran was calculated as a perfused area using Adobe Photoshop CS4 software.

Elisa

Blood from mice was collected by cardiac puncture and centrifuged at 2400 gravitational acceleration for 15 minutes. Collected serum samples were kept at -20 °C until further use. Serum levels of mouse free triiodothyronine (T3) and free thyroxine (T4) were measured and quantified by using an ELISA method according to the manufacture's instruction (MSB 705057 and MSB162146; MyBiosource). In brief, sample or standard samples with known concentrations were placed into a 96 well plate followed by an incubation with conjugate proteins. After several washing, HRP-avidin and substrate solution were applied. Absorbance at 450 nm was detected by a spectrophotometer and free T3 and free T4 concentration were calculated.

RESULTS AND DISCUSSIONS

Adult zebrafish model for study of hypoxic retinopathy. (PAPER I)

Pathological alterations and imbalance of metabolism in retinal vasculature will lead to tissue hypoxia and consequently induce angiogenesis^{207,208}. Tissue hypoxia and angiogenesis will also result in retinopathy, which is a common cause of blindness. Therefore, using an animal model to study the mechanism behind this process is important to develop clinically relevant therapeutic approaches. The non-invasive animal model in the adult zebrafish developed in this thesis aims to mimic hypoxia-induced retinopathy observed in the clinic. This novel model has the main advantage of the ability to validate the efficacy of a water-soluble drugs added to the water.

To construct an adult zebrafish model for studying hypoxia-induced retinopathy, the first task was to consider the required equipment. The setup of the hypoxia facility should be simple and easily controlled in order to obtain accurate and reproducible results. It is of high importance to expose zebrafish to sufficient hypoxic conditions that are equivalent to the level of hypoxia that causes hypoxia-induced retinopathy seen in humans. It is, however, also necessary to ensure that the zebrafish can survive at these low oxygen levels.

Through continuous attempts and adjustments to obtain the optimal level of hypoxia and apparatus set up, a construct an adequate hypoxic state for adult zebrafish was established. To achieve this, we used a lidded but not sealed aquarium perfused with nitrogen to give uniform saturation of the water with nitrogen. The size of the aquarium is of a relatively small size (approximately 1 liter in volume) to enable appropriate administration of water-soluble drugs at later stages of these experiments. To certify the homogeneity of oxygen levels in the water and to decrease the imbalance of pressure produced by the nitrogen gas perfusion, a magnetic stirrer was placed at the bottom of the aquarium covered by a fine net. To accurately monitor and control the appropriate oxygen level in the zebrafish aquarium, the behavior of the fish was carefully observed and oxygen

levels were adjusted accordingly. If their survival rate was low hygiene including water quality was prioritized. By adjusting to acute hypoxia, adult zebrafish developed hypoxia-induced vascular effects on retina after 3 to 10 days of hypoxic exposure.

At the experimental checkpoints, adult zebrafish was immediately fixed and the retina was isolated. The size of eye is around 2-3mm. Hence, proper size of tools and fine techniques under microscope was required. A rapid preparation process of microscopic work and imaging is obligatory to protect the green fluorescence signal produced by the *flil:EGFP* zebrafish. (see Figure 3)

Figure 3. Critical steps of dissecting and analyzing the retinal vasculature in adult zebrafish.

The morphology of the blood vessel marked with green fluorescence can be clearly observed under fluorescent or confocal microscope after flat mounting of the isolated zebrafish retina. The vascular structure of the intact zebrafish retina is highly organized and divided into three regions. First is an artery region containing a central optic artery which consists of nearly 4 to 9 arteries feeding 2 to 4 branches. The second is a surrounding region with one circumferential vein at the edge of the retina. The third is a capillary region connecting to the arterial and venous region.

At 6 days of exposure to hypoxia the retinal vasculature showed structuring tip cells in the arterial region, formation of capillary branches and more disorganized vascular structure in general. When keeping zebrafish longer in the hypoxic environment, the sprouting in arterial region disappeared. It might thus be a transient phenomenon caused by the acute hypoxic condition. For this thesis a quantification method able to evaluate the vascular alterations in zebrafish retina was created to precisely obtain scientifically solid differences in the statistical sense.

Therefore this thesis established, adult zebrafish model for studies of hypoxic retinopathy have certain advantages. The genetic tools available for zebrafish such as morpholinos and transgenic zebrafish are highly developed. Moreover, oral compounds, e.g. water-soluble chemical inhibitors and stimulators, can easily be distributed in the water. Additionally, it is feasible to tightly control the entire experimental process.

There are some limitations of this model. It cannot limit to exposure of hypoxia to individual organs. Regional hypoxia might be more relevant to certain clinical situations. Unfortunately there are few antibodies available, limiting the spectrum of immunohistochemical analysis in zebrafish. Moreover, a highly precise surgical technique to isolate retina is required. Taken together, however, this adult zebrafish hypoxia model of retinopathy provides us with the possibility to perform many experimental applications.

Embryonic zebrafish model for mechanistic study of hypoxic metastasis. (PAPER II)

Hypoxia is a potent inducer for the expression of VEGF, the most important regulatory factor in angiogenesis. Therefore hypoxia induces neovascularization in the tumor environment, which leads to dissemination of tumor cells into the circulation. This pathological angiogenesis is a crucial path in tumor metastasis formation. Although the size of the primary tumor is relatively small during early events of tumor development, tumor invasion and metastasis has already occurred at that time²⁰⁹. Current studies of the tumor metastatic cascade are mainly

focused on the later stages of tumor development, yet the early stage is not detected in animal models. Utilizing the favorable biological characteristics of zebrafish, we were able to develop a metastatic model using embryonic zebrafish. This model allows us to observe single tumor cell dissemination, invasion and metastasis on monolayers of tumor cells in living organisms.

For this development a similar setup as in our adult zebrafish experiment was used (see Paper 1). Several details were modulated depending on the needs for the specific research questions. For example, PTU stock solution should be used for preventing pigment formation in zebrafish embryos during 24-48 hpf. Danieau's solution, the near-isotonic solutions for developing cell transplantation work for the zebrafish embryos, was also used. The oxygen level (7.5%) in water was lower than in the adult zebrafish experiments, due to the fact that embryonic zebrafish tolerate hypoxia better than adults. The higher number (close to 100) of zebrafish embryos placed in hypoxia chamber was required because of a high death rate. Dead embryos exhibit an opaque white. Fortunately, dead embryos had little negative effect on the quality of the water and therefore dead embryos remained in the water until the end of experiments.

The age of 48 hpf *flil:EGFP*-transgenic eggs were used for injection of tumor cells. Nearly 100 fluorescent Dil-labeled human or mouse tumor cells are carefully injected into the embryos. These numbers of tumor cells enable not only ensuring their survival in host, but not disturbing the development of zebrafish embryos due to excessive number of tumor cells. Perivitelline cavity is the preferable location of the transplanted tumor cells where we found. Perivitelline cavity is non-vascularized area so as to restrict the excessive speed of dissemination and invasion of tumor cells from the primary tumor mass into distal parts of the embryo body. After three days tumor implantation, the zebrafish embryos were taken out and examined under fluorescent and confocal microscopy. Implanted tumor cells under hypoxic conditions accomplished dissemination, invasion and metastasis, while the normoxia control group did not show tumor invasiveness.

This model provides an opportunity to study tumor cell invasion and metastasis under hypoxic conditions. The advantages of using zebrafish embryos for a tumor invasion assay are that they are transparent and immunoprivileged. The transparent nature of the fish permits visualization of vascular structures and

tumor dissemination under the microscope. The fact that they are immunoprivileged allows implantation of mammalian tumor cells, including both human and mouse cells. Using morpholinos, target genes in zebrafish embryos are effectively knocked down. Additionally, drugs are dissolved in the water and can easily be administered to block or stimulate biological functions. Furthermore, by using DiI dye to visualize tumors, the observation window is extended up to 7 days. This time frame is sufficient to observe the entire process of tumor dissemination and invasion. The system developed in this thesis permits exposure of the zebrafish embryo to adequate hypoxia levels so that the model can assess hypoxia-induced or VEGF induced angiogenesis and tumor cell metastasis. Therefore, this model is able to test antiangiogenic compounds against new targets found in hypoxia induced angiogenesis and tumor cell migration.

There are some limitations of this model. For instance, the hypoxic area is not localized but affects the entire zebrafish embryo. It is impossible to create a 37°C condition that would be suitable for most mammalian tumors to grow. Zebrafish will not be able to survive at that temperature limiting the number of tumors that can be tested. As mentioned previously training is necessary to acquire the micro-operation skills needed to standardize analyses.

VEGF blockades alter healthy vasculature in mice. (PAPER III)

Anti-VEGF drugs like bevacizumab are commonly used today and are prescribed to cancer patients for treatment of various solid tumors at different locations in the body²¹⁰⁻²¹⁴. Clinicians have observed that these drugs not only have antiangiogenic effects but also produce a wide range of adverse effects including hypertension, renal vascular injury, gastrointestinal perforation and congestive heart failure²¹⁵⁻²¹⁷. The anatomical and structural basis of these adverse effects of anti-VEGF therapy is poorly understood.

This study investigated vascular alterations mediated by the anti-VEGF agents. These agents include an anti-VEGF neutralizing antibody (BD801), an anti-VEGFR1 neutralizing antibody (MF-1) and an anti-VEGFR2 neutralizing antibody (DC101), which binds to VEGF, VEGFR1 and VEGFR2 respectively. The antibodies were administrated to healthy mice systemically for two weeks at

a dose known to block tumor angiogenesis. The comparison of results is shown in Figure 4.

	Endocrine organs			Gastrointestinal tracts										
	Thyroid	Adrenal cortex	Pancreatic islet	Gastric wall	Small Intestine wall	Small Intestine villi	Colon wall	Ovary	Uterus	Liver	Pancreatic acini	Thymus	Renal cortex	
Anti-VEGF-A	***	***	***	*	*	*	*	*	***	*	***	**	***	
Anti-VEGFR-1	***	***	*	*	*	*	*	*	**	*	*	**	***	
Anti-VEGFR-2	ns	ns	ns	ns	ns	ns	ns	*	*	ns	ns	ns	ns	

	Brain				Muscle							
	Glomerulus	Olfactory bulb	Cerebral cortex	Hypothalamus	Cerebellum	Medulla oblongata	Myocardium	Intrinsic lingual	Skeletal muscle	Bone marrow	Retina	Adrenal medulla
Anti-VEGF-A	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Anti-VEGFR-1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Anti-VEGFR-2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

ns : no significant difference * : P < 0.05 ** : P < 0.01 *** : P < 0.001

Figure 4. Impact of Anti-VEGF blockades on vasculature of healthy mice.

The endocrine organs, including the thyroid, adrenal cortex and pancreatic islets, are known to produce high levels of VEGF. Therefore we first examined these organs to analyze vascular alterations by administration of anti-VEGF therapy. Upon anti-VEGF treatment, the thyroid showed a reduced vascular density by almost 60% in the examined whole-mount staining preparations. A similar degree of vascular regression was consistently observed in the other endocrine organs. No significant structural changes were observed by the H&E staining after anti-VEGF treatment. The VEGFR-2 blockade showed an impact on vascular regression in these endocrine organs, while VEGFR1 blockade did not show any vessel regression. These findings pinpointed the important role of VEGF in maintenance of the vascular homeostasis on healthy vessels, including preservation of vascular density. VEGFR-2 was conformed to be the primary functional VEGF receptor involved in vessel maintenance, whereas VEGFR-1 appear to function as a decoy receptor^{94,218}.

Secondly the gastrointestinal (GI) tract, the female reproductive system, renal cortex and glomeruli in the kidney, hepatic sinusoidal in the liver and pancreatic acini were examined in the same way as the endocrine organs. The data in Paper III showed that VEGF and VEGFR2 blockades also reduced vessel density in these organs, although the degree of vessel regression was not as high as what was seen in endocrine organs. Similar to the findings in the endocrine organs,

VEGFR-1 blockade did not produce any vascular regressive effect. Again, no structural changes were observed from the H&E staining.

Finally, the brain, retina, myocardium, skeletal muscles and bone marrow were examined. Importantly, the vasculature in these organs did not show significant response to any anti-VEGF blockades.

As an additional experiment the thyroid was thoroughly investigated as an off-target effect of the administered agent as the vasculature in this organ was affected most by the anti-VEGF treatment. Healthy mice were administrated an anti-VEGF agent for two weeks followed by anti-VEGF cessation. The vessel structure, apoptotic impact on endothelial cells, hypoxia and tissue VEGF level were analyzed. After two weeks of blockade withdrawal vascular regrowth was observed, eventually with nearly normalized vascular density. The results indicate that the vascular regression produced by anti-VEGF treatment is due to decreased endothelial cell survival maintained by VEGF and it is a reversible process. The recovery of the ability of vascular regression in a short time span may have been an effect of tissue hypoxia inducing high expression levels of VEGF to stimulate the angiogenic process. This demonstrates that VEGF is a key factor for vessel maintenance.

Research has demonstrated that systemic delivery of anti-VEGFR component caused vascular regression in various tissues²¹⁹. To study the functional impacts of anti-VEGF treatments on the thyroid, measurements of the blood vessel perfusion and leakiness was performed by using dextran dye in several organs from both anti-VEGF treated and untreated groups. The experimental data demonstrated anti-VEGF agents induced a significant decrease in blood leakage from vessels, but no alteration of blood perfusion, indicating VEGF is an important permeability factor in healthy organs. Subsequently, the serum levels of the thyroid hormone, free T3 and free T4, were detected by ELISA. Free T4 but not free T3 was lower in the anti-VEGF treated group compared to the untreated group. We further examined alterations of vascular fenestrations (openings in the blood vessel wall) in the thyroid upon anti-VEGF treatment, due to the fact that the vascular fenestrations are an important feature for hormone secretion by endocrine organs. As expected, highly fenestrated blood vessels in the untreated group turned to non-fenestrated vessels in the anti-VEGF treated group. Interestingly, more endothelial caveolae was observed in the anti-VEGF treated group. One possible explanation for this observation is that they are

produced to compensate for the suppression of vascular fenestrations to maintain normal hormone secretion in the thyroid.

Taken together, the blockage of the VEGF signaling pathway induces a series of vascular alterations including a reduction in vascular density. Consequently, the vessel and organ functions are in part impaired by anti-VEGF therapies. In a clinical setting anti-VEGF treatment targets tumor sites as well as healthy organs. The effect of anti-VEGF treatment might thus be different between cancer and healthy cells^{216,217}. In addition, cancer patients need to be dosed with anti-VEGF agents for long time, or several cycles with cessation. The adverse effects on healthy organs might be more serious and their function might therefore become irreversible unless some time is allowed for vessel structure to be regained^{220,221}. Tumor sites might develop to be more aggressive due to hypoxia or change of angiogenic profiles are a response to anti-VEGF therapy. These vital points should be addressed using our preclinical mice models in the future.

Discovery of mechanisms underlying circadian clock control of angiogenesis in embryonic zebrafish model.

(PAPER IV)

We investigated the role of the circadian clock in angiogenesis and found there is a coupling of these two factors in the developmental stage of the zebrafish. Importantly we focused on two powerful transcription factors regulating the circadian clock, namely *Bmal1* and *Period2*, and their interaction with an angiogenic factor, VEGF.

The impact of the circadian clock on developmental angiogenesis was studied in Tg(*Flil:egfp*) zebrafish embryos under three different conditions; constant light (LL), constant darkness (DD) and switching between 12 h light and 12 h dark (LD). At the age of 1 hour postfertilization (hpf), the embryos started developing under identical conditions and were visually examined at different time points with regard to their development including viability, phenotype, somite lengths and head angles (two commonly used parameters for staging of the development of zebrafish embryos). Different levels of light exposure did not result in any significant alterations in the studied parameters. However, quantification analysis of developmental vessels showed that LL exposed zebrafish embryos at 72 hpf

relative to 24 hpf possessed decreased development of intersegmental vessels (ISVs) and subintestinal veins (SIVs) as compared with LD exposed zebrafish embryos. DD exposure did not alter ISVs and SIVs as compared with LD exposure. Importantly, the impaired angiogenesis produced by disrupted circadian clock (LL condition) did not affect the development of zebrafish embryos. One possible explanation is that zebrafish takes up oxygen and nutrients through free diffusion instead of supply from functional blood vessels. Another possible explanation may be zebrafish have acquired compensatory mechanisms by having many clock gene homologs. If one clock gene were impaired, another clock gene could compensate through regulation to rescue the defects caused by the failed clock gene.

The circadian control on angiogenesis was further investigated utilizing morpholinos specific to the circadian clock genes *bmal1* and *period2* as a loss of function. Under LD, injection of a *bmal1* morpholino decreased ISV growth while injection of a *period2* morpholino increased ISV growth as compared with controls. Under LL, injection of a *bmal1* morpholino produced no additive effect on the suppression of ISV growth, while injection of a *period2* morpholino abolished the suppression of ISV growth and normalized the ISV growth. This demonstrated that Bmal1 and Period2 displayed opposing effect on angiogenesis. Next, as a gain of function approach, *bmal1* and *period2* mRNAs were injected into the zebrafish embryos. Co-injection of a *bmal1* morpholino with *bmal1* mRNA resulted in nearly normalized ISV defect. LL exposure together with a *bmal1* mRNA injection also restored LL induced ISV inhibition. Furthermore, co-injection of a *period2* morpholino with *period2* mRNA resulted in abolition of *period2* morpholino-mediated ISV growth. These results provided evidence of the specificity of *bmal1* and *period2* morpholinos and confirmation on their opposing angiogenic effects.

To study the role of VEGF in circadian regulated developmental angiogenesis, the zebrafish embryos were injected with combination of *vegf*, *bmal1* or *period2* morpholinos. Injection of *vegf* morpholino alone to zebrafish embryos led to decreased ISV growth in a dose dependent manner. Co-injection of *vegf* and *bmal1* morpholinos resulted in additively decreased ISV growth. On the other hand, co-injection of *vegf* and *period2* morpholinos resulted in slightly increased ISV growth but not an additive effect. Two orally active VEGFR tyrosine kinase inhibitors (TKIs), namely ZN323881 and sunitinib, experimentally displayed the

same effect on regulation of ISV growth as the *veg*f morpholino. Taken together, these results demonstrated that *Bmal1* but not *Period2* mediates VEGF-induced angiogenesis.

The molecular mechanism underlying how *Bmal1* interacts with *veg*f upon regulation of angiogenesis was also studied. The hypothesis was that *Bmal1* may directly target the promoter region of *veg*f. Therefore, a system able to detect the *bmal1* and *veg*f promoter activities using a luciferase reporter system in zebrafish embryos under various light source exposures was employed. Firstly, *veg*f promoter activity was measured under LD or LL condition. As theorized, the *veg*f promoter activity significantly decreased under LL condition. We further investigated *veg*f promoter activity in *bmal1* morpholino-treated zebrafish embryos. Both zebrafish *veg*f and human *veg*f promoter activity were reduced by *bmal1* morpholino treatment indicating that *Bmal1* directly regulates the promoter region of *veg*f. Furthermore, the zebrafish *veg*f promoter activity displayed a dependence on circadian rhythm under LD and DD exposures, while LL exposure diminished zebrafish *veg*f promoter activity and circadian rhythmicity. Measurement of mRNA levels of *bmal1* and *veg*f using qPCR under LD and DD conditions demonstrated circadian rhythmicity, whereas LL exposure disrupted it. These results demonstrate that expression of VEGF and its circadian rhythmicity is regulated by circadian clock gene *bmal1*. Additionally, we analyzed the potential *Bmal1*-binding E-boxes in the promoter regions of *veg*f genes in zebrafish, mice and humans. Deletion of the E-boxes in the promoter region of zebrafish resulted in significantly decreased and even abolished *zveg*f promoter activity. Finally, we performed a chromatin immunoprecipitation assay (a classical method to detect protein and DNA binding) and found that *Bmal1* directly binds to E-boxes in the promoter region of *veg*f.

Moreover, the relationship between the notch signaling pathway and circadian clock on developmental angiogenesis was investigated.

Inhibition of notch signaling resulted in increased but disorganized growth of ISV in the zebrafish embryo. Administration of a *veg*f morpholino reduced the notch inhibition-induced excessive growth of ISV. This demonstrates VEGF regulates notch-induced vascular changes. Importantly the *bmal1* morpholino reduced notch inhibition-induced excessive growth of ISV, while the *period2* morpholino showed no such effect. Even the LL condition resulted in reduction

of notch inhibition-induced excessive ISV growth. The *period2* morpholino together with LL condition showed an increase in the notch inhibition-induced excessive growth of ISV. These results suggested that circadian clock-induced VEGF acts as the driving force for notch-induced angiogenesis.

This paper provides the first evidence that there is a relationship between circadian clock genes and angiogenesis in the developing zebrafish. It would be very important to further investigate the role of circadian clock genes in diseases – in particular cancer development, tumor growth and metastasis, and drug resistance in cancer patients, as well as healthy vascular maintenance.

CONCLUSIONS

This thesis elucidates VEGF-mediated angiogenesis in physiological and pathological conditions using zebrafish and mice models.

In conclusion:

1. It is possible to develop zebrafish models using embryos and adults to study mechanisms of angiogenesis under hypoxia.
2. The embryonic zebrafish model demonstrates the feasibility of studying tumor metastasis at the early stage of metastatic events as well as hypoxia-induced metastasis in real time.
3. The adult zebrafish model permits evaluation of angiogenic mechanisms in hypoxia-induced retinopathy.
4. Inhibition of VEGF signaling in healthy mice alters the mature vasculature and in part impairs function in some organs.
5. The main receptor for VEGF signaling that maintains healthy vasculature is VEGFR2.
6. The circadian clock has an important role in the regulation of developmental angiogenesis in an embryonic zebrafish model.

Circadian clock genes *bmal1* and *period2* are involved in developmental angiogenesis, and *bmal1* directly regulates VEGF production.

FUTURE PERSPECTIVES

This thesis' work has paved new conceptual and methodological avenues for understanding the fundamental mechanisms of angiogenesis in physiology and pathology. Based on our zebrafish cancer metastatic model, we can reasonably speculate that this model could potentially be used to predict the metastatic potential and risk in a given cancer type in patients. To date, clinical assessments of cancer invasion and metastasis are largely based on observational data in patients and pathological properties of malignant cells per se. However, a functional *in vivo* model to study the metastatic potentials in living animals has been lacking. Thus our findings provide a functional system that can detect cancer cell behavior at the single cell level. Consistent with this notion, we have correlated the cancer invasion in the zebrafish model and the metastatic disease in a limited number of cancer patients. Our preliminary findings show that highly metastatic cancer types can be accurately predicted in our zebrafish model²²². It would be interesting to expand this type of studies in the future and hopefully this functional assay could be routinely used in the clinic in combination with observational clinical data when needed. Future studies based using this zebrafish metastasis model should also be able to recapitulate the tumor microenvironment in which various host cells can be included in the metastatic assays. To this end, we have shown that the tumor-associated macrophages, especially the M2 macrophage subpopulation, are crucial for promoting cancer metastasis²²². In line with this we plan to develop multi-color-coded system to label various cells including tumor cell, macrophage, stromal fibroblast, endothelial cell, and perivascular cells with different color to study their role in facilitating cancer metastasis. Genetic zebrafish models in combination with color-coded isolated cells should then be used. With all this information available, our zebrafish cancer model can also be used to evaluate the role of various signaling molecules affecting metastasis formation. Accordingly, one of the most important advantages of our system is that we can study the metastatic events that the early stage of cancer development.

In the retina neovascularization zebrafish model, we have demonstrated and analyzed hypoxia-induced retinal angiogenesis in a non-invasive adult system. To the best of our knowledge, this is the first non-invasive and non-genetically

manipulated retinal angiogenesis model in adult animals. Exposure of adult zebrafish in hypoxic water can be easily performed and zebrafish tolerate well to hypoxic environment. With this system, it would be interesting to screen for potential drugs that interfere with retinal neovascularization. As hypoxia is very common driving force for neovascularization, our model system is clinically relevant. This model can possibly be further developed for use under diabetic conditions. We hope that it will provide a reliable platform for discovery of new drugs that target retinal angiogenesis and these potential drugs will be used for treatment of millions of patients with diabetic retinopathy.

The clinical implication of “off-tumor” targets of anti-VEGF drugs is to understand the mode of actions of these drugs and their potential side effects. Particularly, for cancer patients who have pathological changes in one tissue where the anti-VEGF drug targets most vasculature it is of great importance. Particular attention should be given adverse events to optimize safety for patients. We believe that our findings in mouse models accurately predict the antiangiogenic drug effects in human cancer patients. Indeed, hypertension and hypothyroidism are common side effects of anti-VEGF drugs and these clinically observed adverse effects correlate well with these drug-induced vasculature changes in thyroid and kidney.

The groundbreaking discovery that the circadian clock is of importance in the regulation of angiogenesis has stimulated our thinking to expand our efforts to pathological conditions such as cancer. It would be pivotally important to study if the disruption of clock genes in mice could cause drug resistance. If so, we need to develop a spate line of medication in combination with antiangiogenic drugs to improve therapeutic efficacy. Moreover, timing of any anti-cancer agent could be optimized. This interesting and important possibility warrants future study and validation.

Taken together, our findings presented in this thesis have paved many different new lines of possibilities for treatment of cancer and non-malignant diseases. If any of our approach would be successful in the future, it would be beneficial for millions of patients.

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PAPER I

Hypoxia-induced retinopathy model in adult zebrafish

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Hypoxia-induced vascular responses, including angiogenesis, vascular remodeling and vascular leakage, significantly contribute to the onset, development and progression of retinopathy. However, until recently there were no appropriate animal disease models recapitulating adult retinopathy available. In this article, we describe protocols that create hypoxia-induced retinopathy in adult zebrafish. Adult *flk1:EGFP* zebrafish are placed in hypoxic water for 3–10 d and retinal neovascularization is analyzed using confocal microscopy. It usually takes 11 d to obtain conclusive results using the hypoxia-induced retinopathy model in adult zebrafish. This model provides a unique opportunity to study kinetically the development of retinopathy in adult animals using noninvasive protocols and to assess therapeutic efficacy of orally active antiangiogenic drugs.

INTRODUCTION

Vascular pathology and metabolic alterations often lead to tissue hypoxia, which triggers several host responses including vasodilation, elevated hematopoiesis and angiogenesis^{1–3}. In type 2 diabetes, retinopathy as a common complication usually results from hypoxia-induced neovascularization due to abnormal metabolism⁴. However, development of a clinically relevant and noninvasive hypoxia-induced retinopathy model in animals is a challenging task, which hampers assessment of the efficacy of potential therapeutic drugs.

To study the impact of hypoxia-induced pathological angiogenesis on retinopathy development, we have recently developed novel methods of hypoxia-induced retinopathy in adult zebrafish⁵. As *in vivo* angiogenesis-dependent disease models, zebrafish have several advantages over other existing disease models in mammals^{6–8}. These include availability of genetically manipulated zebrafish strains, functional knockdown of specific genes by the morpholino technology, easy and low-cost maintenance of the zebrafish facility, and the capacity to breed a large number of zebrafish within a relatively short period of time. Using adult *flk1:EGFP* zebrafish⁹, we developed a hypoxia-induced retinopathy model in which retinal neovascularization occurs at the predicted region of the optic disc⁵. In this model, adult zebrafish are placed in a specifically designed aquarium and water oxygen levels can be automatically monitored using an oxygen sensor. After exposure to hypoxic water for a few days, retinal neovascularization is readily detected under a fluorescent microscope.

During exposure to hypoxia, it is convenient to add orally active, small chemical compounds to the hypoxic water so the antiangiogenic activity can be detected relative to controls⁵. As the retinal vasculature in the adult zebrafish consists of a monolayer network of microvessels that are composed of highly organized arterioles, capillaries and venules, hypoxia-induced vessel growth in different regions can be easily quantified by simply counting the number of sprouts, new branches and vascularization areas.

In contrast to our zebrafish model, other existing mammalian retinopathy models¹⁰ use invasive procedures such as laser-induced damage to trigger retinal neovascularization¹⁰. However, these invasive procedures are far from being clinically relevant, because retinopathy-associated pathology in clinical settings rarely involves mechanical damage other than metabolically or immunologically linked tissue hypoxia. In this regard, our current protocol is highly

relevant to the clinical situation as compared with other existing models. This hypoxia-induced retinopathy model in adult zebrafish allows researchers to study the mechanisms of hypoxia-induced angiogenesis *in vivo*; to study the vessel formation, vascular remodeling, and leakiness under pathological settings; to correlate vascular changes with disease development; to screen potential therapeutic agents that are orally active; and to assess the beneficial effects of known antiangiogenic agents for the treatment of retinopathy. It should be emphasized that it is often inconvenient to expose adult mammals to a hypoxic chamber to induce retinopathy. Moreover, unlike the existing mammalian retinopathy models, detection of retinopathy in zebrafish does not require immunohistochemical staining, owing to the availability of *flk1:EGFP* and *flk1:EGFP* strains¹¹. Our protocols do not require sophisticated facilities, and skillful experimenters should be able to implement this method in their research.

Advantages and limitations

Key advantages:

- It is inexpensive to obtain a large number of adult zebrafish.
- Maintenance of zebrafish is simple and convenient.
- Generation of genetic strains by deletion or overexpression of specific genes can be easily accomplished using available tools.
- Genetically manipulated zebrafish strains that are defective or have acquired functions of certain gene products are available.
- Genetic strains that express reporter genes in particular cell types are also available.
- Addition of orally active chemical stimulators or inhibitors to the aquarium water enables analysis of intervention of these compounds on physiological and pathological processes.
- Hypoxia setups for zebrafish are simple and can be tightly controlled. Oxygen levels in the fish aquarium can be accurately adjusted using an automated monitor and control system.
- The turnover time for experiments is relatively short.

Limitations:

- It is difficult to obtain local tissue or organ hypoxia rather than systemic exposure to hypoxia.
- Owing to the limitation of available antibodies against zebrafish proteins, it is difficult to perform immunohistochemical analysis using zebrafish tissues.

Figure 1 | Hypoxia setup for adult zebrafish. (1) Breeding insert net; (2) oxygen regulator; (3) adult hypoxia aquarium; (4) solenoid valve; (5) oxygen electrode; (6) air stone; and (7) magnetic bar. Electrical wires are marked as black lines. The compressed nitrogen source is shown as a blue tank, and the nitrogen-containing gas tubes are marked with blue.

•Owing to the small size of zebrafish, skillful and careful operations are required to perform experiments.

Experimental design

Creation of hypoxic conditions. Creation of a hypoxic aquarium for zebrafish might be achieved by either perfusing nitrogen gas into water directly or into the air inside a sealed aquarium. However, perfusion of nitrogen gas into the aquarium air may create high pressure and lead to high death rates in zebrafish. In our experimental design, we chose to perfuse nitrogen gas into the water of the aquarium (see EQUIPMENT SETUP for further information). As shown in **Figure 1**, a 1-liter aquarium was chosen for convenience and for reducing the cost of drug treatment. Smaller aquaria may present difficulties of accurately adjusting oxygen levels in the H_2O . The aquarium is designed as an automated unit, which automatically controls nitrogen gas perfusion depending on oxygen levels in the water. Oxygen levels are accurately measured by an oxygen electrode connected to an oxygen regulator and the nitrogen gas supplier. Despite the automation of the hypoxic aquarium, pilot studies should be conducted before experimentation by placing a few zebrafish into the aquarium for at least 3 d. In case of unexpected early death of zebrafish during experimentation, the oxygen regulator should be recalibrated.

Replicates and controls. Owing to the variation of individual zebrafish in response to hypoxia, a relatively large number of zebrafish should be included in each experimental group. To obtain statistically meaningful results, typically 12 adult zebrafish should be used in each experiment; at least two experiments are recommended in order to repeat the findings. To design each experiment optimally, all necessary controls should be included. The age, sex and physical status of all zebrafish under hypoxic and normoxic conditions should be identical. The normoxic aquarium, as a control, should be similarly designed as the hypoxic chamber except lacking nitrogen gas supply. Both aquaria should be placed in the same environment, as temperature, moisture and oxygen content in the room may affect experimental results.

Drug treatment. When designing drug treatment experiments, chemical solvents used as vehicles are added to control

groups under normoxic and hypoxic conditions. Thus, for each experiment testing the effects of a drug on hypoxia-induced retinal neovascularization, there are three controls: vehicle in normoxia, drug in normoxia and vehicle in hypoxia. The least toxic vehicle for a particular drug should be chosen. If ethanol or DMSO is used as vehicle, we strongly recommend having sufficiently strong stock solutions so that the final concentration of the vehicle is less than 0.1% in water; this will minimize the unspecific effects of the vehicle. For example, in a previous study we used anti-VEGF drugs such as sunitinib to study the effects of VEGF on hypoxia-induced retinal neovascularization⁵. In that study, sunitinib was dissolved in citrate buffer at 8 mg ml⁻¹ (15 mM) and diluted 30,000 times to reach a final concentration of 0.5 μ M in the hypoxic water.

Results analysis. In the assessment of experimental findings, both left and right eyes of each zebrafish are used to validate results. However, variations of retinal vascularization between the two eyes of each zebrafish may exist. Dissection of zebrafish eyes and isolation of the retina are extremely challenging tasks and demand technically skillful micro-operations under a stereoscope. To ensure high success rates, it is strongly recommended that the investigator should practice on a daily basis and avoid intake of adenosine receptor activators such as coffee, which may increase uncontrolled hand movements. Once the retinal samples are prepared, we recommend examining the results as soon as possible as EGFP fluorescence undergoes progressive quenching. At the stage of results analysis, similar regions of hypoxia- and normoxia-exposed retinal vasculature should be used for comparative evaluation because the architecture and structure of retinal vessels vary in different regions (**Figs. 2 and 3**). It is important to quantify intercapillary distance, vessel density, and new branches and sprouts using the same magnification; preferably, 15–25 images from at least six individual fish from each experiment should be quantified to reach a justified conclusion.

Figure 2 | Critical steps of dissecting and analyzing the retinal vasculature in adult zebrafish. Adult *flr1:EGFP* zebrafish exposed to normoxia and hypoxia were anesthetized with tricaine and killed. All the fish were immersed immediately in 4% PFA for 24 h. Fish eyes were enucleated and placed into a Petri dish containing 1x PBS. Using fine surgical forceps and scissors, fish retinas were carefully isolated and examined under a fluorescent microscope.

Figure 3 | Hypoxia-induced retinal angiogenesis.

(a–d) The retinal vasculature of adult zebrafish is divided into three regions: the arterial region (AR), which consists of I–IV grade of arteries⁵; the capillary area (CA); and the collecting veins (ColV). For the quantification of angiogenic responses, each region should be quantified separately. (a) High-magnification view of the area defined by a blue box in b. (b) Confocal micrograph of EGFP-positive vessels in the retina of a zebrafish exposed to normoxic water.

(c) Confocal micrograph of EGFP-positive vessels

in the retina of a zebrafish exposed to hypoxic water (10% air saturation) for 6 d. (d) High magnification of the area defined by a blue box in c. Under the hypoxic conditions shown in our protocol, CA (between dashed red lines), spanning the 350 μm between the arterial region and collecting veins, usually shows considerable angiogenic responses, whereas AR and ColV exhibit minor alterations. For quantification of intercapillary distance, a midline (purple line in b and c, at the position of 175 μm from ColV) should be drawn and intercapillary distances are measured crossing this line, as shown in a and d (purple bidirectional arrows). Arrows point to sprouts and arrowheads indicate endothelial tips. Yellow dots in b and c indicate branch points. CV, circumferential vein. Scale bars in a and d, 25 μm . Scale bars in b and c, 100 μm .

MATERIALS

REAGENTS

- 1× PBS (prepared in house or purchased from Hyclone, cat. no. SH30028.02)
- Colorless nail polish
- Compressed nitrogen (97%; AGA Gas)
- Distilled water (dH_2O)
- Ethyl 3-aminobenzoate methanesulfonic acid salt (98%, tricaïne; Sigma-Aldrich, cat. no. E10521)
- Paraformaldehyde (PFA, prills, 95%; Sigma-Aldrich, cat. no. 441244-1KG)
- **CAUTION** Highly toxic. Small solid PFA particles or fumes can easily be inhaled during preparation or handling of the 4% solution. Always work with gloves and never open PFA-filled containers or tubes outside a fume hood.
- Vectashield mounting medium (Vector Laboratories, cat. no. H-1000)
- Zebrafish (*Tg(fli1:EGFP)*¹, 3–24 months old; ZFIN)
- Sodium hydroxide (NaOH; Sigma, cat. no. S5881)
- Hydrochloric acid (HCl; Merck, cat. no. 3.0317-1)

EQUIPMENT

- Adobe Photoshop CS3 (or later versions; Adobe)
- Aquatic oxygen monitor and regulator (made in house, see EQUIPMENT SETUP and further information on the following homepage: <http://www.mbl.ku.dk/JFSteffensen/OxygenTemperatureMonitorRegulator/>. Alternatively, can be purchased from Loligo, cat. no. OX10000)
- Bacterial culture dish (10 cm; Corning, cat. no. 391-1517)
- BD falcon 50-ml polypropylene conical tubes (BD Biosciences, cat. no. 358206)
- BD Micro-Fine and 1-ml BD U-100 insulin syringe (0.33 mm; BD Biosciences, cat. no. 305930)
- Confocal microscope (Nikon D-eclipse C1; Nikon)
- Confocal software (EZ-C1 3.9 Nikon digital eclipse; Nikon)
- Dissecting stereomicroscope (Wild M3Z; Leica)
- Eppendorf polypropylene conical tubes (2 ml; VWR International, cat. no. 211-2120)
- Fish transfer nets (Fridhems Akvarier)
- Forceps superfine tips (Dumonts finest tip, AgnTho's, cat. no. 11254-20)
- Glass beaker (100 ml, Boro 3.3)
- Magnet bar (4 cm; VWR International, cat. no. 442-4527)
- Magnet stirrer with heating (VMS-A; VWR International, cat. no. 442-0185)
- Microscope coverslips (Thermo Scientific, cat. no. 631-0135)
- Microscope slides (Thermo Scientific, cat. no. 631-1303)
- Microsoft Office Excel 2003 (or later version; Microsoft)
- Nonheated magnet stirrer (VS-C7-2 EU-1; VWR International, cat. no. 442-0551)
- Plastic containers (185 mm × 85 mm × 75 mm; Aqua-Schwarz)
- Plastic lid for zebrafish hypoxia chamber (200 mm × 100 mm × 14 mm; Aqua-Schwarz)
- Plastic pipette tips (10–1,000 μl ; Sarstedt, cat. no. 70762.100)
- Reusable adhesive (Sense; Clas Olsson, cat. no. 26-888)
- Scalpel blades (no 11, Allgaier instrumente; AgnTho's AB, cat. no. 02-040-011)
- Scalpel holder (no. 3, Allgaier instrumente; AgnTho's AB, cat. no. 02-030-030)
- Spatula/microspoon (VWR International, cat. no. 231-1354)

- Surgical scissors (2 and 6 mm, Vanna; AgnTho's AB, cat. no. 15000-03)
- Tissue needle, straight tip (VWR International, cat. no. 238-6250)
- Zebrafish spawn insert (175 mm × 80 mm × 63 mm; Aqua-Schwarz)
- Programmable LED indicator (PR Electronics, cat. no. 5714)
- Solenoid valve (Bürkert)
- OxyGuard mini oxygen electrode (OxyGuard, cat. no. D161SV)
- ImageJ software

REAGENT SETUP

4% PFA (wt/vol) Dissolve 20 g PFA powder in 500 ml of 1× PBS by warming the solution to 60 °C under constant stirring or in a water bath for about 16 h. The 4% PFA fixative solution can be kept at 4 °C for a maximum of 2 weeks.

▲ CRITICAL Fresh solution is crucial to the success of experiments. If PFA solution has been stored longer than 2 weeks, discard the old solution and prepare a fresh batch.

Tricaïne stock (0.05%) For convenience, a 25× stock solution in dH_2O (0.05%) should be prepared. This stock solution is diluted to a final concentration (0.002%) immediately before use. Tricaïne powder (50 mg) is dissolved in 100 ml dH_2O to obtain the stock solution. Prepare 2-ml aliquots in plastic vials and store them at –20 °C until use. **▲ CRITICAL** Tricaïne is unstable at low concentrations (2× concentration or lower) in aqueous solutions. Do not prepare a stock solution at a low concentration.

EQUIPMENT SETUP

Hypoxia aquarium setup The aquarium is a standard 1-liter plastic chamber (185 mm × 85 mm × 75 mm) with a plastic lid containing a hole on one side to allow installation of the nitrogen supply. The hole is located at the bottom corner of the short side of the aquarium (Fig. 1). Insert a gas tube through the hole, which is sealed with plastic cement to prevent leakage. An air stone is connected to the end of the gas tube and is placed at the bottom on the opposite side of the aquarium. The other end of the tube is connected to the solenoid regulator valve that controls nitrogen perfusion from the compressed nitrogen bottle. Place a magnetic bar at a central position at the bottom of the aquarium, which is placed on a nonheated magnetic stirrer. Switch on the stirrer and rotate the magnetic bar gently. Create another hole (2 mm) on the opposite side of the aquarium; this hole should be located close to the lid. Through this hole, a wire connected to the oxygen electrode is inserted. Place a breeding insert (mesh size: 2 mm) into the aquarium and leave the bottom several millimeters from the top of the air stone. The breeding insert prevents physical contact between zebrafish and the magnetic stirring bar. Place the oxygen electrode at the bottom of the breeding insert at a position such that the electrode tip is far from the air stone. Use plastic cement to seal the space between the wire and hole. Create a hole (5 mm in diameter) in the lid; this hole releases the air pressure within the aquarium.

Oxygen regulator setup The oxygen regulator is essentially a programmable LED indicator equipped with a solenoid valve from Bürkert and an OxyGuard mini oxygen electrode as described on the following website: <http://www.mbl.ku.dk/JFSteffensen/OxygenTemperatureMonitorRegulator/>.

PROTOCOL

PROCEDURE

Adaptation to hypoxic environment ● TIMING 36 h

1| Add 0.9 liters of prewarmed water (28.5 °C) to a hypoxia aquarium and calibrate the system to display 100% air saturation in normoxic water and to show the measurement indicator at the zero position when short circuited ('0 cal' on the bottom of the front panel of the oxygen regulator) (Fig. 1).

▲ **CRITICAL STEP** The water level in the aquarium should not exceed 80% of the total aquarium depth. Leave approximately 20% air space for controlling oxygen exchange.

2| (Optional) Dissolve orally active chemical compounds in a desirable vehicle such as DMSO, ethanol or water (or, in the case of sunitinib, citrate buffer) as a stock solution.

3| (Optional) Add the chemical compounds to the aquarium water at concentrations that functionally intervene with hypoxia-induced host responses. In the case of sunitinib, add 30 µl of 8 mg ml⁻¹ sunitinib stock solution to the 0.9-liter aquarium water (from Step 1).

4| Transfer 10–12 healthy adult *flil:EGFP* transgenic zebrafish (3 to 24 months old) to the normoxic water in the aquarium. Stir water at a relatively slow rotation speed to ensure sufficient circulation of water in the entire aquarium (~150 r.p.m.).

▲ **CRITICAL STEP** Water temperature in the hypoxia aquarium should be equilibrated to the same temperature as the water in which the zebrafish are maintained. Larger magnetic bars are recommended for better mixing at slow rotation speeds.

▲ **CRITICAL STEP** Also place a group of adult zebrafish in normoxia (regular water) in an aquarium to serve as a control. If chemical compounds are being tested and have been added to the hypoxia aquarium at Step 3, add the appropriate amount of the same drug and vehicle to normoxia control aquaria (see Experimental design for further information on controls). Thus, three controls—vehicle in normoxia, vehicle in hypoxia and drug in normoxia—should be included in each experiment.

5| Turn on the nitrogen gas (approximately 3 atm) in the hypoxia aquarium and perfuse nitrogen gas through the air stone into the water. Set the limit to 20% and hysteresis to 0.2 on the oxygen regulator (Fig. 1).

▲ **CRITICAL STEP** The nitrogen air stone should be kept clean so that nitrogen gas is released evenly by forming small bubbles. The nitrogen pressure should not be too high to prevent suffocation of fish.

6| Keep the fish at 20% air saturation for 8–12 h; follow this by decreasing air saturation to 15% as shown on the oxygen regulator.

7| Keep the fish at 15% air saturation for 5–6 h, then decrease the air saturation to 13% as shown on the oxygen regulator.

! **CAUTION** At this time point, almost all zebrafish should show signs of having hypoxia by exhibiting water-surface breathing and elevated respiration frequencies. As a result of hypoxia, some zebrafish may die. It is crucial to remove dead fish immediately from the aquarium to prevent infection in surviving fish.

8| Keep the fish at 13% air saturation for 5–6 h; follow this by decreasing air saturation to 11.5% shown on the oxygen regulator.

9| Maintain the fish at 11.5% air saturation for 5–6 h, then decrease air saturation to 10% (820 p.p.b. at 28.5 °C) as shown on the oxygen regulator. This time point is considered day 0 for exposure to hypoxic water.

7 TROUBLESHOOTING

Hypoxia exposure period and zebrafish dissection ● TIMING 3–12 d

10| Inspect the zebrafish in the hypoxia aquarium several times per day and ensure that the nitrogen pressure is steadily maintained at 10% air saturation. Should any fish die, remove them immediately from the aquarium.

7 TROUBLESHOOTING

11| At the end of the hypoxia exposure period (usually 6–12 d), transfer zebrafish using a net to 50 ml of 0.004% tricaine and keep them in tricaine for at least 10 min after cessation of gill movements. Fix the dead fish by submerging 1–4 fish in 30 ml of 4% PFA pre-equilibrated to 4 °C in a 50-ml Falcon tube and incubate at 4 °C for at least 24 h.

Figure 4 | Critical steps of dissecting and flattening the adult zebrafish retina. (1) Create a small hole at the circumferential side of the cornea by a sharp syringe needle. (2) Cut off the cornea along the limbal basis with a pair of surgical microscissors. (3) Extrude the lens with microsurgery forceps. (4) Insert a sharp syringe needle between the sclera and the retina and create a small hole. (5) Expand the space between the retina and sclera with a needle and a pair of microscissors. (6) Tear off the sclera and choroid with forceps. (7) Position the retina in the middle of a glass slide with the inside facing up. (8,9) Cut the retina disc three or four times, mount it in Vectashield and cover with a glass cover slip.

12| Wash the zebrafish with 1× PBS twice and transfer them to a Petri dish (10 cm in diameter) containing 20 ml of 1× PBS (**Fig. 2**). Dissociate fish heads from the rest of the body using a pair of fine surgical scissors. With scissors and forceps, gently disassociate the operculum and gills. The eyeballs connected to the optic nerve should now be apparent when inspecting the fish from the ventral side. Release the eyeballs by cutting the optic nerves as close to the eyeballs as possible.

■ **PAUSE POINT** Whole fish or fish eyes fixed in 4% PFA can be stored in 1× PBS at 4 °C for up to 2 weeks before dissection and flat-mounting of the retinas.

Zebrafish retina flat mounting ● **TIMING** 15–60 min per retina

13| In a plastic Petri dish (10 cm in diameter) containing 1× PBS, position the eye with the cornea upward using microsurgery forceps. Use a sharp syringe needle to create a wound at the circumferential side of the cornea. Carefully cut off the cornea along the limbal basis with a pair of surgical microscissors, then extrude the lens. Flip over the eye to place the optic nerve stump upward. Gently insert a sharp needle between the sclera (outer layer of the eye) and the retina and separate the two without damaging the retina. Expand the space between the retina and sclera with the needle and gently tear off the sclera and choroid (brown color) with forceps (**Figs. 2 and 4**).

14| Position the retina in the middle of a glass slide with the inside facing up. Carefully cut the retina disc three or four times from the edge toward the center to divide it evenly into three or four ‘petals’ of a flower-shaped sheet (**Figs. 2 and 4**). Remove excessive water with a piece of tissue paper and add a drop of Vectashield on the retina. Place a glass cover slide (22 mm × 22 mm) on the retina and seal the edges with nail polish.

■ **PAUSE POINT** Mounted retinas can be stored horizontally in the dark at –20 °C for up to 2 weeks before microscopic examination.

! **CAUTION** From the time when zebrafish are killed, the fluorescent EGFP signal starts to degrade and quench. This degradation process can be delayed by fixation/mounting. For best results, we recommend microscopically analyzing eyes within 2 weeks after killing the zebrafish.

Imaging ● **TIMING** 2–6 h

15| To obtain an overview of a whole petal of the retina, which is often used to show the general changes in the retinal vasculature (for example, in presentations or publications), illuminate the sample at low magnification (×4 objective) with blue light of approximately 488 nm and use the correct filter (i.e., EGFP or FITC) to obtain EGFP-positive signals using a fluorescent microscope. Alternatively, scan EGFP-positive signals at 488 nm using a confocal microscope (see **EQUIPMENT**). When using a confocal microscope at low magnification, the thickness of a scanned tissue layer in focus is high, indicating that the total number of scanned layers is relatively low (i.e., one or two). Therefore, image acquisition time is greatly shortened. For a more detailed image analysis, scan the samples at higher magnifications (×10, ×20, ×40 or ×63 objectives). At high magnification, images can reveal detailed information, including vascular branch points, sprouts, endothelial cell tips and filopodial extensions. The resolution should be at least 1,024 × 1,024 (**Figs. 2 and 3**). For more information on imaging and quantification parameters, see **Box 1**.

BOX 1 | QUANTIFICATION OF RETINAL ANGIOGENESIS

1. Analyze the vasculature of dissected retinas mounted on glass slides using confocal microscopy at $\times 4$, $\times 10$ or $\times 20$ magnification objectives (see Step 15).
2. Take serial micrographs of retinal vessels covering the vascular region from the central optic artery to the circumferential vein. Take at least 2–5 pictures from each retina, depending on the magnification. A calibrated scale bar indicating a fixed length (e.g., 100 μm) should be included in all images. Such a scale bar can be obtained by capturing a picture of a microscope ruler under the same microscope settings as used to capture images of the zebrafish retina. These serial micrographs of each retina allow definition of the arterial regions, capillary areas (anastomosis between arteries and collecting veins) and circumferential veins (Fig. 3). Hypoxia-induced vessel sprouts, branch formation, endothelial cell tips and networks can be detected in the capillary area (Fig. 3).
3. Define the capillary area to be used for quantification as an area spanning 350 μm from the beginning of the collecting veins toward the arterial region (red dashed lines in Fig. 3). First rotate the image until the circumferential vein is perfectly horizontal. Next, if using Adobe Photoshop, define a fixed selection box with dimensions equal to the width of the image and a height of 350 μm , using the scale bar as a reference. Set the selection box at the beginning of the collecting veins (Fig. 3) and crop the selected area. Using ImageJ, you should first crop the image at the beginning of the collecting veins (Fig. 3), and then crop the image again such that its height is precisely 350 μm (use the scale bar as a reference). The resulting cropped image should then have the same width and height (350 μm) and is defined as the capillary area.
4. Draw a horizontal midline (175 μm from both ends of the capillary area defined in the previous step) through the capillary area. In Adobe Photoshop, the line is drawn using the line tool, and the distance is measured using the calibrated size bar in each image as a reference. In ImageJ, the distance can be measured in the same way, and the image can be cropped at the midline rather than by drawing an actual line.
5. Measure intercapillary distance as shown in Figure 3 using the straight line selection tool followed by the measure tool in ImageJ. Measure all intercapillary distances crossing the midline from 15 to 25 images for each experimental condition and calculate the average.
6. With Adobe Photoshop, calculate the capillary density by selecting greens (or if the image is black and white, select highlights) using the color range tool. The number of selected pixels as seen from the histogram window is noted. This value is divided by the total number of pixels in the area, and multiplied by 100% to give the capillary density in percent. Calculate the average of 15–25 images per group.
7. Manually count all branching points in the capillary area of each micrograph shown as yellow dots in Figure 3. Calculate the average of 15–25 images per group.
8. New vascular sprouts are defined as blunted vascular branches that have not connected to other vessels (Fig. 3, arrows). Manually count all sprouts in the capillary area of each micrograph. Calculate the average of 15–25 images per group.
9. Endothelial cell tips are defined as thin fibers, as shown in Figure 3 (arrowheads). Manually count endothelial cell tips in the capillary area of each micrograph. Calculate the average of 15–25 images per group.
10. Either Adobe Photoshop or ImageJ (which is free, open-source software) can be used for analysis. Although quantification procedures in ImageJ of vessel sprouts, branch formation and endothelial tips are exactly the same as described above using Adobe Photoshop, measurement of the vascular area is achieved by converting green fluorescent signals into white signals on a black background using the ‘color-split channels’ command in ImageJ followed by the selection of only the green channel. Use the ‘binary’ command followed by the ‘analyze particles’ command to measure the capillary area. The sum of the area of all particles is divided by the total number of pixels in the image and multiplied by 100% to give the capillary area in percent. Calculate the average of 15–25 images per group.

▲ CRITICAL STEP The retina is a relatively thick tissue and contains autofluorescent cells. Images acquired with fluorescent microscopy may therefore contain a relatively high, noisy background. We recommend that readers analyze the samples using confocal microscopy, if available.

? TROUBLESHOOTING

16| Analyze the data using the Adobe Photoshop software program. Vessel density, capillary vascular area, intercapillary distance and vascular branches can be accurately quantified as detailed in Box 1 (Figs. 2 and 3).

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
9, 10	All zebrafish die after just a few days	The oxygen level may be too low	Calibrate the oxygen electrode to make sure it shows an accurate oxygen level. Have nitrogen flow at a rate where overshooting is at a minimum. It is critical to watch the breathing rate of each fish rather than simply monitoring the oxygen readers
		The water may be contaminated	Make sure the water is of high quality (free from pollutants, and with optimal conductivity and pH). Water quality can be maintained relatively well throughout the experiment by not feeding fish the day before and during the experiment, as well as by removing dead fish immediately
		The fish are exhausted from swimming	Make sure the rotation speed of the magnet is not too high for fish that are forced to swim along the water current
		The fish are fighting with each other	Have a sufficient number of fish in the tank from the beginning (not fewer than six) and remove fish that are strongly dominating the others
		The lid is sealed too tightly	High air pressure can kill fish. Make sure the ventilation hole is not blocked
15, 16	Lack of angiogenic response	The oxygen level is not sufficiently low	Calibrate the oxygen electrode to make sure it shows the true oxygen level. Lower the oxygen level to 8.5–9.0% air saturation. Carefully monitor fish behaviors such as breathing rates and surface breathing
		Insufficient exposure time	Increase hypoxia exposure time
		Samples are stored too long	Analyze freshly prepared samples

● TIMING

Steps 1–9, Adaptation to hypoxic environment: 36 h
Steps 10–12, Hypoxia exposure period and zebrafish dissection: 3–12 d
Steps 13 and 14, Zebrafish retina flat mounting: 15–60 min per retina
Steps 15 and 16, Imaging: 2–6 h

ANTICIPATED RESULTS

According to the detailed experimental procedure, described in sequential steps above, the expected result of hypoxia-induced angiogenesis in adult *flt1:EGFP* zebrafish is shown in **Figures 2** and **3**. Under normoxia, the architecture of retinal vasculature is highly organized as a single layer of vascular network in which the optic artery is located at the center of the optic disc. About 4–9 arteries branch from the optic artery and are evenly distributed in each optic disc; these secondary arteries are further segregated 4–5 times before anastomosing with protrusions from the circumferential vein, which is located around the edge of the optic disc. The arterial-venous anastomosis area consists of microcapillaries that form double-ended capillary plexuses. Under hypoxia, subtle changes in the arteries, capillaries and circumferential veins can be easily detected by fluorescent microscopy. Kinetic studies show that capillary sprouts begin on day 3 and the formation of a vascular network becomes obvious on day 6 after exposure to hypoxia⁵. After day 6, hypoxia-induced endothelial tip and capillary branch formation are only present in the capillary area (**Figs. 2** and **3**), which can be easily quantified as numbers of branches and sprouts, intercapillary distance and vascular area. However, prolonged exposure to hypoxia after day 6 results in modest changes in the retinal vasculature⁵. As distribution of the optic vasculature in each zebrafish varies, at least six retinas should be used for assessing results. Typically, 4–9 grade I arterial branches from the central optic artery can be found in the zebrafish retina^{5,12}. However, the number of sub-branches (grade II–IV) from grade I vessels may vary considerably depending on the number of the grade I vessels. In general, fewer grade I vessels would result in more branching grade II–IV vessels to cover the retinal disc. Therefore, for quantification, it is highly recommended to choose the same region of the optic disc for comparison (**Box 1**), and six retinas should be used. For confocal imaging analysis, we recommend mounting the isolated retina samples onto glass slides with cover slips using ×4, ×10, ×20 or ×40 magnification objectives. **Figure 3** provides an example of hypoxia-induced retinal angiogenesis captured with the ×10 magnification objective.

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PAPER II

Hypoxia-induced metastasis model in embryonic zebrafish

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Hypoxia facilitates tumor invasion and metastasis by promoting neovascularization and co-option of tumor cells in the peritumoral vasculature, leading to dissemination of tumor cells into the circulation. However, until recently, animal models and imaging technology did not enable monitoring of the early events of tumor cell invasion and dissemination in living animals. We recently developed a zebrafish metastasis model to dissect the detailed events of hypoxia-induced tumor cell invasion and metastasis in association with angiogenesis at the single-cell level. In this model, fluorescent DiI-labeled human or mouse tumor cells are implanted into the perivitelline cavity of 48-h-old zebrafish embryos, which are subsequently placed in hypoxic water for 3 d. Tumor cell invasion, metastasis and pathological angiogenesis are detected under fluorescent microscopy in the living fish. The average experimental time for this model is 7 d. Our protocol offers a remarkable opportunity to study molecular mechanisms of hypoxia-induced cancer metastasis.

INTRODUCTION

Invasion and metastasis are the hallmarks of malignant disease, and understanding the molecular mechanisms underlying cancer metastasis may define new therapeutic targets for anticancer drug development. Hypoxia is known to significantly facilitate tumor invasion and metastasis in association with angiogenesis¹. However, mechanisms of hypoxia-induced tumor invasion and metastasis remain poorly understood. One of the initial steps of the metastatic cascade involves the dissemination of malignant cells from the primary site². Tumor cell dissemination in cancer patients can occur at the early stage of tumor development, when primary tumors are relatively small³. Unfortunately, current cancer detection methods at both clinical and preclinical levels are not able to image early events of tumor cell dissemination from primary sites.

Owing to the transparent and immunoprivileged nature of zebrafish embryos, we have recently developed a tumor invasion and metastasis model in association with hypoxia-induced angiogenesis^{2,4}. This metastatic model allows us to detect tumor cell dissemination at the single-cell level. Using this method, we demonstrated that VEGF- or hypoxia-induced angiogenesis facilitate tumor cell dissemination^{2,4}. Implantation of fluorescently labeled mouse or human tumor cells into the perivitelline space of zebrafish embryos, which are subsequently placed in a hypoxic aquarium, allows us to study early events of metastasis while primary tumors are relatively small. This is the first animal model to study detailed events of cancer metastasis in relation to tumor hypoxia and angiogenesis. This protocol is complementary to other existing models that are primarily designed to study the later events of the metastatic cascade, including the formation of metastatic niches and the regrowth of tumors in distal tissues and organs^{2,5,6}. Unlike other models, monitoring of tumor cell dissemination with respect to angiogenesis and hypoxia in embryonic zebrafish can be achieved in living animals, which allows kinetic analysis of tumor cell dissemination and formation of distal metastatic niches in the same animal.

The general advantages of using zebrafish to study pathological angiogenesis and disease development are described by Cao *et al.* in this issue⁷. Additionally, hypoxia-induced metastasis in zebrafish embryos provides an outstanding opportunity to define signaling components in the host, which facilitates tumor invasion, dissemination and metastasis using specific morpholino intervention technology. It should be emphasized that genetic manipulations of crucial components involving the hypoxia-induced host responses could also create global hypoxia in the host. For example, deletion of the von Hippel-Lindau tumor suppressor gene in zebrafish results in a systemic hypoxic response which recapitulates the key aspects of Chuvash polycythemia in humans⁸. These genetically altered hypoxic zebrafish models offer alternative options for studying the hypoxic effect on tumor angiogenesis and metastasis in zebrafish.

This protocol has broad applications for studying the mechanisms underlying cancer cell metastasis, invasion and spreading; for defining new molecular targets controlling tumor cell migration and hypoxia-regulated angiogenesis; for assessing antimetastatic effects of known anticancer drugs; and for discovering potential new drugs for the treatment of metastatic diseases. In addition to its use in the assessment of therapeutic efficacies of monotherapies, it is anticipated to be a powerful model to study the therapeutic effects of combination therapy containing two or more therapeutic molecules. This zebrafish metastatic model is complementary to existing mouse tumor and metastatic models, allowing the study of different aspects of cancer metastasis.

Advantages and limitations

Key advantages:

In addition to the general advantages and limitations of using zebrafish as an *in vivo* model to study hypoxia-induced angiogenesis (see the companion article by Cao *et al.*⁷), there are several other advantages of using zebrafish embryos for studying metastasis. These include:

PROTOCOL

- Effective knockdown of gene expression by morpholino technology in zebrafish embryos allows readers to study functions of specific gene products.
- The transparent nature of zebrafish embryos allows visualization of vascular changes and tumor cell dissemination under hypoxia and normoxia in living fish.
- Immunoprivilege of zebrafish embryos allows implantation of mammalian tumor cells, including human and mouse tumor cells⁴.

Limitations:

- As the hypoxia chamber is designed for the whole embryo, it is difficult to create hypoxia only in the tumor tissue.
- As most mammalian tumors grow at 37 °C, it is difficult to study the process of xenograft tumor growth at the optimal temperature.
- Microinjection of tumor cells into the perivitelline space of a large number of zebrafish embryos is a tedious procedure and requires highly skillful micro-operations.

Experimental design

Aquaria. The general setup and design for the hypoxic and normoxic aquaria are described in the companion article in this issue⁷. For zebrafish embryos, we recommend using a nylon mesh (mesh size <0.25 mm) to separate zebrafish embryos from the magnetic stirring bar, as fish embryos are unable to swim against water currents.

Tumor cells. Monolayers of human, mouse or other mammalian tumor cells should grow in culture to subconfluency (about 70–80%) to ensure that they are at the proliferating phase. Although genetically manipulated tumor cells that express fluorescein-activated proteins such as red fluorescent protein or yellow fluorescent protein may be used for *in vivo* tracing, we do not recommend using enhanced green fluorescent protein (EGFP)-labeled tumor cells if *fli1:EGFP* or *flk-1:EGFP* zebrafish strains^{9,10} are used. For convenience, we use 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), which labels cell membranes for a relatively long time (~2 weeks) without losing color⁴.

Embryo production. While growing tumor cells, at least 5–6 pairs of adult zebrafish of breeding age should be placed in the breeding chamber (one pair per chamber) to ensure production of 200–300 eggs. We highly recommend choosing as many healthy embryos produced by the same pair of adult zebrafish as possible, as they are usually at the identical age.

Tumor cell injection. Approximately 100 DiI-labeled tumor cells should be injected into the perivitelline space and should not be implanted into the yolk sac. As considerable variation exists

between individual zebrafish embryos in response to hypoxia, a relatively large number of zebrafish embryos should be included in each experimental group; in each experimental and control group, 100 zebrafish embryos should be implanted with tumor cells. Implantation of tumor cells into the perivitelline space of embryos at 48 h post fertilization (h.p.f.) is technically challenging; thus, a skillful and experienced person should perform injections. The investigator should not drink coffee or other caffeine-enriched beverages in order to avoid uncontrolled hand movement.

Embryo selection. Even after tumor cell implantation, fish embryos should undergo further selection under fluorescent microscopy to ensure that tumor cells are located only within the perivitelline space.

Experimental conditions. To obtain statistically meaningful results, at least 50 embryos should be used in each experimental and control group, as it is expected that some embryos will die during experimentation. Once placed in the hypoxic chamber, we do not recommend opening the lid and disturbing embryos until they are harvested for analysis. For analysis, each individual zebrafish embryo in both hypoxic and normoxic groups should be photographed using fluorescent microscopy. These data should be digitally deposited in Adobe Photoshop, ImageJ or an equivalent program for further analysis.

Drug treatment. In drug treatment experiments, chemical solvent is used as vehicle and should have as low toxicity as possible. Dissolve the orally active chemical compound such as sunitinib in a desirable solution such as distilled water in high concentrations to prepare the stock solution. According to the appropriate final concentration of the drug in the hypoxia and normoxia aquaria, add the appropriate volume of the stock solution. As an example, if the stock concentration is 10 mM, to reach a final concentration of 1 μ M in 1 liter of hypoxic or normoxic aquaria embryo water, the drug should be diluted 10,000 $\times$; accordingly, 100 μ l of stock should be added to the water.

Controls. In all experimental designs, appropriate controls should be used under normoxia and hypoxia. It is known that tumor cells, even though derived from the same origin of the same cell type, may have different potentials to trigger angiogenic responses and to metastasize. Thus, if applicable, tumor cells with high or low metastatic potentials from the same type of cancer cell should be considered to serve as positive and negative controls. If genetically manipulated tumor cells are used, it is essential to include two control groups under normoxia and hypoxia: (i) nonmanipulated wild-type tumor cells; and (ii) vector or mock-manipulated tumor cells.

MATERIALS

REAGENTS

- 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Invitrogen, cat. no. D3899)
- 1-Phenyl-2-thiourea (PTU; Sigma-Aldrich, cat. no. P7629)
- Agarose (SeaKem LE, cat. no. 50004)
- Calcium chloride (CaCl₂, Sigma-Aldrich, cat. no. C8106)
- Distilled water (dH₂O)
- Dulbecco Modified Eagle's Medium (DMEM (Sigma-Aldrich, cat. no. D6546)
- Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, cat. no. D8537)

- Ethyl 3-aminobenzoate methanesulfonic acid salt ((98% purity) tricaine; Sigma-Aldrich, cat. no. E10521)
- FBS (Hyclone, Thermo scientific, cat. no. SV30160.03)
- HEPES buffer (1 M in water; Sigma-Aldrich, cat. no. 83264)
- L-glutamine solution (200 mM, Gibco, cat. no. G7513)
- Magnesium sulfate (MgSO₄, Sigma-Aldrich, cat. no. M7506)
- Nail polish, colorless
- Nitrogen gas (100%, AGA GAS AB)
- Penicillin-streptomycin solution (Gibco, cat. no. 15070-063)
- Potassium chloride (KCl; Sigma-Aldrich, cat. no. P5405)
- Sodium chloride (NaCl; Merck, cat. no. 1.06404.1000)

- T241 mouse fibrosarcoma cell line (ATCC)
- Transgenic (*flil:EGFP*) zebrafish were obtained from ZFIN (Zebrafish Model Organism Database; <http://www.zfin.org/>)
- Trypsin solution (2.5% (wt/vol), 10×, Gibco, cat. no. 15090-046)
- Vectashield mounting medium (Vector Laboratories, cat. no. H-1000)

EQUIPMENT

- Photoshop CS3 or later versions (Adobe)
- ImageJ software
- Bacterial culture dish (10 cm; Corning, cat. no. 391-1517)
- BD Falcon polypropylene conical tubes (50 ml; BD Biosciences, cat. no. 358206)
- CO₂ incubator (Thermo electron corporation, model 311)
- Confocal microscope (Nikon D-eclipse C1, Nikon)
- Confocal software (EZ-C1 3.9 Nikon digital eclipse, Nikon)
- Costar cell culture plates (48-well; Corning, cat. no. 3548)
- Dissecting stereomicroscope (Wild M3Z, Leica)
- Eppendorf microinjector (FemtoJet 5247, Eppendorf, cat. no. 5247000.013)
- Eppendorf polypropylene conical tubes (2 ml, VWR International, cat. no. 211-2120)
- Fish transfer nets (Fridhems Akvarier)
- Fluorescent software (NIS-Elements D 3.0, Nikon)
- Forceps superfine tips (Dumont's finest tip, AgnTho's AB, cat. no. 11254-20)
- Heraeus BK 800 refrigerated incubator (Thermo Scientific, cat. no. 50120350)
- Magnet bar (4 cm; VWR International, cat. no. 442-4527)
- Magnet stirrer with heating (VMS-A, VWR International, cat. no. 442-0185)
- Manipulator (MM33-Right, Märzhäuser Wetzlar, cat. no. 00-42-101-0000)
- Microloader tips (0.5–10 µl; Eppendorf, VWR, cat. no. 5242 965.003)
- Microscope cover slips (Thermo Scientific, cat. no. 631-0135)
- Microscope slides (Thermo Scientific, cat. no. 631-1303)
- Microsoft Office Excel 2003 or later version (Microsoft)
- Nonfilamentous borosilicate glass capillaries needles (1.0 mm in diameter; World Precision Instruments, cat. no. 1B100-4)
- Nonheating magnet stirrer (VS-C7-2 EU-1, VWR International, cat. no. 442-0551)
- Oxygen regulator (OxyReg, Loligo, cat. no. OX10000)
- P-97 Flaming/Brown-type micropipette puller (Automated Scientific, cat. no. SU-P-97)
- Pasteur pipette (150 mm, wide, Bergman Labora AB)
- Pipette pump (10 ml, Pi-pump, VWR, cat. no. 612-3756)
- Plastic containers (185 mm × 85 mm × 75 mm and 200 mm × 100 mm × 130 mm, Aqua-Schwarz)
- Plastic lid for zebrafish embryo hypoxia chamber (200 mm × 100 mm × 14 mm, and 245 mm × 145 mm, Aqua-Schwarz)
- Plastic pipette tips (10–1,000 µl; Sarstedt, cat. no. 70762.100)
- Reusable adhesive (Sense, Clas Ohlson, cat. no. 26-888)
- Pipette (1 ml; Sarstedt, cat. no. 861251.001)
- Tissue needle (straight tip, VWR, cat. no. 238-6250)
- Zebrafish spawn insert (175 mm × 80 mm × 63 mm, Aqua-Schwarz)

REAGENT SETUP

Tricaine stock (0.05% (wt/vol)) For convenience, a 25× stock solution in dH₂O (0.05% (wt/vol)) should be prepared. To prepare the 25× stock, tricaine powder (50 mg) is dissolved in 100 ml dH₂O; prepare 2-ml aliquots in plastic vials and store at –20 °C until use. This stock solution is diluted to a final concentration (2×, 0.004% (wt/vol)) immediately before use. **▲ CRITICAL** Tricaine is unstable

PROCEDURE

Tumor cell injection in zebrafish embryos ● TIMING ~3 d

1| Day 0: place a breeding insert inside a 1 liter breeding aquarium, fill the aquarium with aquarium water and put one male and one female adult *flil:EGFP* zebrafish inside the breeding insert. Let the zebrafish mate overnight; fertilized eggs can be harvested the following morning.

2| Day 1: the next morning, collect eggs from fertilized *flil:EGFP*-transgenic zebrafish and incubate them at 28.5 °C in the Danieü's solution (1%, dilute stock 1:100 in dH₂O) for 24 h.

? TROUBLESHOOTING

Figure 1 | Hypoxic setup for the embryonic zebrafish aquarium. (1) Nitrogen gas tank; (2) oxygen regulator; (3) embryonic hypoxia aquarium; (4) valves; (5) oxygen electrode; (6) air stone; (7) magnetic bar; and (8) nylon mesh.

at low concentrations (2× concentration or lower) in aqueous solutions. Do not make a stock solution at a low concentration.

Danieü's aquarium water (30×)

Prepare 1 liter sterile dH₂O containing 1.74 M NaCl, 21 mM KCl, 12 mM MgSO₄, 18 mM CaCl₂ and 150 mM HEPES buffer. This inorganic aquarium water can be kept at 22 °C for up to 12 months.

1-Phenyl-2-thiourea (PTU, 10×) Dissolve PTU powder in dH₂O to a final concentration of 2 mM. **▲ CRITICAL** It usually takes several hours to dissolve PTU at room temperature with aid of a magnet stirrer. Once completely dissolved, PTU solution can be kept at 4 °C for a maximum of 2 weeks.

Agarose gel preparation To prepare a solution containing 2% (wt/vol) agarose, add 0.8 g agarose to 40 ml distilled water in a glass beaker and heat the solution in a microwave oven until all agarose particles are dissolved. Add 20 ml of the agarose solution into a Petri dish (10 cm diameter) and let it harden. About 15–20 min later, warm the rest of the solution in a microwave oven and add the solution on the top gel layer. Before solidification, place a mold in the center of the dish and leave it embedded in the agarose gel while it hardens. About 30 min later, remove the mold and add ~20 ml of 1% (vol/vol) sterile Danieü's aquarium water to immerse the gel. The agarose gel plate can be stored at 4 °C for ~30 d until use. Agarose plates should be pre-equilibrated to 26–28 °C before microinjection.

Preparation of Dil stock solution According to the manufacturer's instructions, add 97% (vol/vol) ethanol to the powder compound to constitute a concentration of 20 mg ml^{–1}; mix the solution to ensure that the Dil compound is completely dissolved. This stock solution can be stored in 5-µl aliquots at –20 °C.

DMEM cell culture medium preparation (FBS-DMEM) To prepare this medium, supplement the DMEM with 10% (vol/vol) FBS. Also add 1% (vol/vol) L-glutamine and 1% (vol/vol) penicillin-streptomycin. Store this medium at 4 °C. Before use, it should be pre-equilibrated to room temperature or, preferably, to 37 °C.

EQUIPMENT SETUP

Hypoxia aquarium for zebrafish embryos A hypoxia chamber for zebrafish embryos is created using a 1-liter standard plastic aquarium with a lid (Fig. 1). In general, the hypoxia chamber setup for zebrafish embryos is the same as for adult zebrafish (see Cao *et al.*⁷), with the exception that the bottom of the insert chamber is made of fine nylon mesh (mesh size <0.25 mm).

PROTOCOL

3| *Day 2*: add the PTU stock solution into the fish water to constitute a final concentration of 0.2 mM (10× dilution) and incubate the embryos for an additional 24 h.

! CAUTION PTU is used for preventing pigment formation in zebrafish embryos and is highly neurotoxic and carcinogenic. Thus, this chemical compound must be handled with a great caution in a chemical fume hood. PTU dishes and containers must be properly marked and handled only with gloves, and PTU waste should be kept in specific containers, which are disposed of separately.

4| *Day 2 (continued)*: while incubating zebrafish embryos with PTU for 24 h (incubation is usually started in the morning) prepare tumor cells labeled with DiI dye. Use monolayers of tumor cells cultured in 75 cm³ culture flasks in DMEM supplemented with 10% (vol/vol) FBS, 1% (vol/vol) L-Glu and 1% (vol/vol) penicillin-streptomycin (FBS-DMEM, see REAGENT SETUP) at approximately 70–80% confluency. Add 1 µl of 20 mg ml⁻¹ DiI stock into 10 ml of 1× DPBS (pre-equilibrated to room temperature) to achieve a final concentration of 2 µg ml⁻¹. Discard the conditioned medium from the flasks and add 5 ml of DPBS pre-equilibrated to room temperature to the flask shake gently and remove it. Add 10 ml of the DiI labeling solution into each of the 75 cm³ culture flasks containing monolayers of tumor cells (**Fig. 2**); follow this by incubating cells at 37 °C for 30 min.

▲ CRITICAL STEP It is important to add the DiI into prewarmed 1× DPBS because DiI dye can solidify in cold DPBS. This is a crucial step to prevent blockage of capillary needles during the microinjection process of tumor cells into zebrafish embryos.

5| Collect all the medium containing the labeled tumor cells that become detached from the flasks as a result of DiI labeling and add it to a 50-ml Falcon tube; centrifuge at 1,000g for 3 min and discard the supernatant.

? TROUBLESHOOTING

6| Wash labeled cells with 1× DPBS twice: add ~5 ml of DPBS into the tube containing cell pellet and resuspend the cells in DPBS by pipetting up and down using a 1-ml pipette; centrifuge at 1,000g for 3 min and discard the supernatant. Repeat this step one more time.

▲ CRITICAL STEP To remove all free dye particles from the cell suspension, labeled tumor cells should be washed thoroughly.

7| Add 2 ml of 10% (vol/vol) FBS-DMEM (both fresh and pre-equilibrated to room temperature) and resuspend all the labeled cells with help of a pipette as described above; add the cell suspension into a new 75 cm³ flask containing 13 ml of fresh 10% (vol/vol) FBS-DMEM without DiI, followed by incubation at 37 °C overnight.

8| *Day 3*: Use two pairs of micro-surgical forceps to dechorionate 48 h.p.f. embryos from Step 6. Hold the chorion with one forceps, and, with the help of the other forceps, grip the chorion and tear it apart (**Fig. 2**).

? TROUBLESHOOTING

9| Anesthetize the dechorionated zebrafish embryos by transferring them to fish water containing 0.004% (wt/vol) tricaine and wait for ~2 min.

10| Place up to ten embryos in each well and add 0.004% (wt/vol) tricaine water to each well of the 2% (wt/vol) modified agarose gel plate to precisely fill the wells. Excessive water can be removed with a Pasteur pipette.

▲ CRITICAL STEP If an excessive amount of water is used, zebrafish embryos can float outside the agarose

Figure 2 | Critical stages for tumor cell implantation into embryonic zebrafish and for analyzing tumor cell dissemination. *flt1:EGFP* zebrafish embryos at 48 h are dechorionized. Meanwhile, human or mouse tumor cells growing as monolayers are labeled with DiI dye and resuspended at an optimal concentration for microinjection. Approximately 100–150 tumor cells in 5 nL are injected into the perivitelline space of each zebrafish embryo. Tumor-implanted zebrafish embryos are incubated in normoxic and hypoxic water for 3 d. Dissemination of tumor cells can be detected using fluorescent microscopy at the single-cell level. Tumor cell (red) invasion and migration in association with tumor angiogenesis (green) or co-option of surrounding vasculatures in the host can be revealed using a two-channel fluorescent microscope.

well, leading to empty wells. Additionally, excessive water can result in difficulties in positioning the zebrafish embryos at a fixed position for injection.

11| To prepare tumor cells from Step 7 for implantation, remove the medium and wash attached monolayers of tumor cells twice with 1× DPBS by adding 5 ml of 1× DPBS into the flask, shaking the flask very gently and then removing the DPBS.

12| Add 2 ml of 0.25% (wt/vol) trypsin pre-equilibrated to room temperature into the flask containing attached monolayer of tumor cells and incubate at room temperature for 30 s. Remove the trypsin, tap the flask a few times very gently to help the cells to detach and stop trypsin activity by adding 2 ml of 10% (vol/vol) FBS-DMEM. Resuspend the tumor cells in the medium by pipetting up and down. Count the tumor cells under a phase contrast microscope. Centrifuge tumor cells at 1,000g for 5 min and resuspend cells at a concentration of 20–30 cells per nl in 0.5–1% FBS-DMEM (e.g., FBS-DMEM as prepared in REAGENT SETUP, but with 0.5–1% FBS instead of 10% FBS).

▲ **CRITICAL STEP** It is crucial to stop trypsin activity by adding serum-containing cell culture medium. Otherwise, viability of cells can be markedly reduced during the counting of cells.

7 TROUBLESHOOTING

13| Place the cell suspension on ice during the entire microinjection procedure.

14| Pull nonfilamentous borosilicate glass capillary needles using a micropipette puller. Load the microcapillary glass needle with 4 µl of cell suspension and connect the needle to the micromanipulator. Point the needle tip to the injection site and gently insert the needle tip into the perivitelline space of the zebrafish embryo. Inject 5 nl of the cell suspension containing approximately 100–150 cells into the perivitelline space of each embryo from Step 10 (Fig. 2).

7 TROUBLESHOOTING

15| Transfer the injected zebrafish embryos to PTU water. The death rate of zebrafish embryos after tumor cell injection is ~5%.

Hypoxia exposure period ● TIMING 3 d

16| Fill the hypoxia chamber for zebrafish embryos with 1% (vol/vol) Danieü's water to the two-thirds level of the aquarium and calibrate the oxygen electrode using the same procedure described in adult zebrafish hypoxia model (Cao *et al.*). Switch on the nitrogen gas and set the air saturation level to 7.5% as shown on the oxygen regulator (Fig. 1).

17| If drug treatment is being tested, add the chemical compound to aquarium water at the concentration that functionally intervenes with hypoxia-induced host responses (optional; see Experimental design).

18| When the hypoxia reaches a steady-state level, transfer tumor-implanted zebrafish embryos from Step 15 to the hypoxia chamber and close the lid. Incubate up to 100 tumor cell-implanted zebrafish embryos in the hypoxia chamber at 28 °C.

▲ **CRITICAL STEP** A group of tumor-implanted zebrafish embryos should be placed in normoxia (regular water) to serve as a control.

▲ **CRITICAL STEP** Make sure that the lid is not completely sealed in order to avoid high pressure. If the lid is completely sealed, zebrafish embryos may die of anoxia.

19| Use a medium-sized magnet bar to mix the water and create a mild water current within the chamber to ensure that nitrogen gas is evenly distributed.

20| Maintain embryos in the hypoxia chamber for 3 d.

▲ **CRITICAL STEP** The death rate is quite high at this hypoxia level; therefore, it is very important to closely monitor the chamber during the course of the entire experiment. Unlike in adult zebrafish experiments, there is no need to remove dead zebrafish embryos. To ensure that all zebrafish embryos are evenly exposed to the same hypoxic level, embryos should be swirling gently around in the chamber.

21| After 3 d, transfer the tumor cell-implanted zebrafish embryos in water exposed to normoxia or hypoxia onto the surface of a 2% (wt/vol) agarose gel with help of a wide-tip Pasteur pipette for further analysis.

▲ **CRITICAL STEP** Analysis should be carried out immediately once the zebrafish embryos are placed on the agarose gel, as zebrafish embryos can only live for a few minutes in this condition.

PROTOCOL

Imaging ● TIMING 6–9 h

22| At room temperature, cut a piece (1 cm × 1 cm) of 2% (wt/vol) agarose gel and place it on a glass slide. Use a Pasteur pipette to pick up a zebrafish embryo and carefully place it onto the gel cushion. Remove excessive water and add a small drop of 0.04% (wt/vol) tricaine onto the embryo.

▲ **CRITICAL STEP** If embryos are not completely anesthetized, increase the concentration of tricaine. Imaging should be performed immediately after anesthesia to reduce the risk of embryo death due to liquid evaporation.

23| Collect an embryo with a wide-tip Pasteur pipette and transfer it onto a glass slide; remove the excess water around using a paper tissue. If the paper tissue touches the embryo, the embryo will get dry and cannot be used for imaging. Put a small drop of Vectashield on the embryo, place a cover glass on it and seal the edges of the cover slide with nail polish. Keep the glass slide in a horizontal position in the dark for a short while until the nail polish dries and then transfer immediately to -20°C .

■ **PAUSE POINT** Mounted embryos at the horizontal position can be stored in dark at -20°C for up to 2 weeks before microscopic examination.

24| Use fluorescent microscopy to analyze the number of disseminated tumor foci and cells. Perform imaging at low magnification ($\times 4$ objective) using the ultraviolet-activated filter at the wavelength of 488 nm. The fluorescent light filter allows excitement of EGFP in zebrafish embryos. Use filters for green fluorescence to visualize zebrafish embryo vasculature and filters for red fluorescence to image implanted tumor cells labeled with DiI. To illuminate the DiI-labeled tumor cells in zebrafish embryos, use green light at the wavelength of 555 nm. It is essential to capture green fluorescent-positive and red fluorescent-positive signals at the same position on the embryos in order to associate DiI-labeled tumor cells with blood vessels by superimposing green and red signals. Typically, for each zebrafish embryo, two different sets of images from the head region and the tail region are collected as the separate green fluorescent-positive signals, the red fluorescent-positive signals, and the merged signals. All disseminated tumor cell signals in each embryo should be counted. At least 13–20 embryos in each experimental group should be used to obtain statistically significant values.

25| Use three-dimensional (3D) confocal microscopy to study angiogenesis and metastasis in more detail. Confocal low magnification ($\times 4$ objective) can be used to image the whole body to get an overview of the tumor cell dissemination pattern, and higher magnifications ($\times 20$ and $\times 40$ objectives) are suitable for studying intratumoral and peritumoral angiogenesis and also for precise localization of disseminated cells and foci inside the zebrafish embryo body. Use a 488-nm laser to scan the zebrafish embryo vasculature and a 543-nm laser to scan DiI-labeled implanted tumor cells. To achieve a high-quality 3D image, each embryo should be scanned in eight to ten layers and each layer should be scanned six times to remove the background and get a sharp picture (Fig. 3).

26| To quantify the number of disseminated cells and foci, count all the cells and foci that have disseminated from the tumor mass toward the fish embryo head and tail using fluorescence software, ImageJ or any other kind of useful software program. For further data analysis, use Excel or similar software to calculate the average number of disseminated foci and cells. In addition, to measure the distance of metastasis, choose the focus or cell that lies furthest away from the tumor mass and measure the distance between that focus and the primary tumor. The programs and software mentioned above can also be used for this purpose. Adobe Photoshop can be used to measure and analyze the ratio of tumor vessel density relative to size and tumor volume; see the accompanying paper⁷ for more details.

Figure 3 | Dissemination and quantification of disseminated mouse T241 fibrosarcoma cells. Approximately 100 DiI-labeled mouse T241 fibrosarcoma cells were injected into the perivitelline space of each 48 h.p.f. *flil:EGFP* zebrafish embryo. The tumor cell-implanted zebrafish embryos are exposed to normoxic or hypoxic water at 28°C for 72 h. Green color represents zebrafish embryo blood vessels, red color represents DiI-labeled tumor cells and yellow color shows overlapping positive signals. (a,b) Tumor cell dissemination and metastases under hypoxic conditions (a) and angiogenesis under normoxia (b) were detected using confocal microscopy. Scale bar applies to both panels a and b. (c) Percentage of zebrafish carrying disseminated tumor cells (total number of embryos studied was 13 for hypoxia and 15 for normoxia) under normoxia and hypoxia were quantified. All DiI-positive cells located outside the primary tumor mass were manually counted as disseminated cells or foci using the software mentioned in Step 26. (d) The average number of disseminated foci from primary tumor implanted in zebrafish embryos (13 for hypoxia and 15 for normoxia) were quantified. (Adapted from ref. 4 with permission.) Error bars indicate the standard deviation; asterisks stand for $P < 0.001$ and show the significant difference between the two groups.

7 TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
2	Too few eggs	Nonproductive breeding pairs	Choose a few healthy, well-fed breeding pairs of zebrafish to ensure enough eggs
5	Tumor cells remain attached after 0.5-h incubation with labeling medium	Highly adherent cell line	Release cells by resuspending them in the labeling medium using a 1-ml pipette tip. Avoid using trypsin at this stage
8	Embryos are destroyed during dechorionization	Inexperienced and less skillful personnel	Practice by starting with a large number of embryos and choose perfect ones for injection
12	Formation of cell clusters	Dead cell clumps	Add 0.5% FBS to the medium to reduce trypsin-induced cell death
14	Blockage of injection capillaries	Formation of clumps and too much cell debris	Load the microcapillary needle with the desired volume of cell suspension, pipette up and down a couple of times, remove the suspension and load the needle again or increase cell density and reduce the injection volume

● TIMING

Step 1, Breeding (day 0): 15 h

Step 2, Incubation of embryos in Danieü's solution (day 1): ~24 h

Steps 3–7, Incubation of embryos in 0.2 mM PTU solution (day 2): ~24 h

Steps 8–15, Dechorionization and tumor cell injection in zebrafish embryo (day 3): 2–3 h per 100 embryos

Steps 16–21, Hypoxia exposure period: 3 d

Steps 22–26, Imaging: 6–9 h

ANTICIPATED RESULTS

Hypoxia-induced dissemination of tumor cells in embryonic zebrafish

Using this protocol, dissemination of DiI-labeled (T241, fibrosarcoma) tumor cells can be detected at single-cell level using a fluorescent microscope (stage 5 in **Figs. 2** and **3**). Moreover, hypoxia-induced tumor angiogenesis (green color) in relation to dissemination of tumor cells (red color) can be monitored using a two-channel confocal image analysis. Notably, a majority of disseminated tumor cells are associated with the vasculature, demonstrating the essential role of angiogenesis in tumor invasion and metastasis. As the primary tumor remains at a relatively small size during the entire experimental procedure, hypoxic exposure is unlikely to generate an uneven gradient that causes tumor cells to be superficially invasive owing to co-option to surrounding healthy vasculature. **Figure 3** shows an example of the expected results of metastasis under hypoxia using DiI-labeled mouse fibrosarcoma cells with quantification of metastases.

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PROTOCOL

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PAPER **III**

Anti-VEGF– and anti-VEGF receptor–induced vascular alteration in mouse healthy tissues

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Systemic therapy with anti-VEGF drugs such as bevacizumab is widely used for treatment of human patients with various solid tumors. However, systemic impacts of such drugs in host healthy vasculatures remain poorly understood. Here, we show that, in mice, systemic delivery of an anti-VEGF or an anti-VEGF receptor (VEGFR)-2 neutralizing antibody caused global vascular regression. Among all examined tissues, vasculatures in endocrine glands, intestinal villi, and uterus are the most affected in response to VEGF or VEGFR-2 blockades. Thyroid vascular fenestrations were virtually completely blocked by VEGF blockade, leading to marked accumulation of intraendothelial caveolae vesicles. VEGF blockade markedly increased thyroid endothelial cell apoptosis, and withdrawal of anti-VEGF resulted in full recovery of vascular density and architecture after 14 d. Prolonged anti-VEGF treatment resulted in a significant decrease of the circulating level of the predominant thyroid hormone free thyroxine, but not the minimal isoform of triiodothyronine, suggesting that chronic anti-VEGF treatment impairs thyroid functions. Conversely, VEGFR-1-specific blockade produced virtually no obvious phenotypes. These findings provide structural and functional bases of anti-VEGF-specific drug-induced side effects in relation to vascular changes in healthy tissues. Understanding anti-VEGF drug-induced vascular alterations in healthy tissues is crucial to minimize and even to avoid adverse effects produced by currently used anti-VEGF-specific drugs.

angiogenesis | vascular homeostasis | vessel regression | antiangiogenic therapy | off-tumor targets

The antiangiogenic concept for treatment of solid tumors was proposed by Judah Folkman 41 y ago (1). In this hypothetical paper, Folkman wrote that “one approach to the initiation of ‘anti-angiogenesis’ would be the production of an antibody against tumor angiogenic factor.” Today, the most commonly used anti-VEGF drug, bevacizumab (avastin) (2), is a humanized neutralizing antibody against tumor-derived VEGF, validating Folkman’s early prediction.

Tumors produce various angiogenic factors and cytokines to induce angiogenesis that is essential for tumor growth. Among these tumor-derived factors, VEGF, also called VEGF-A, is a key angiogenic factor that induces angiogenesis, vascular permeability, and tortuosity (3, 4). VEGF displays these broad vascular functions via binding and activation of its specific receptors, VEGFR-1 and VEGFR-2, mainly expressed in vascular endothelial cells, although other cell types may also express these receptors (5–8). Abundant experimental data demonstrate that VEGFR-2 is the primary functional receptor that transduces both angiogenic and vascular permeability signals whereas VEGFR-1 may function as a decoy receptor (9, 10). It should be emphasized that, under physiological and pathological conditions, VEGFR-2 is expressed not only in angiogenic vessels, but also in quiescent vasculatures in various tissues (11). The broad distribution of VEGFRs in quiescent vasculatures in various healthy tissues suggests that the VEGF–VEGFR signaling system is essential for maintenance of

vascular homeostasis. In some tissues, such as endocrine organs including adrenal glands, thyroid, and pancreatic islets, the endothelium in these vasculatures remains fenestrated and VEGF is crucial for maintenance of vascular fenestrations (12–14). Kamba et al. reported that systemic delivery of tyrosine kinase inhibitors (TKIs) containing the anti-VEGFR component caused vascular regression in various tissues (15). However, TKIs have broad targets, and it was unclear whether blocking VEGFR signaling was solely responsible for the vascular phenotype.

In the present study, we chose to use VEGF- and VEGFR-specific blockades that do not target other signaling systems. Additionally, we have also distinguished the significance of VEGFR-1- and VEGFR-2-mediated signaling pathways in maintenance of vascular homeostasis in various tissues and organs. Given the fact that bevacizumab is the most commonly used antiangiogenic drug in oncology (16–21), our findings using these specific blockades may be directly translated into clinical relevance concerning the global impact of these drugs in cancer patients and the structural basis of adverse effects. Thus, the information provided in our study could be potentially useful for development of new therapeutic strategies to minimize or avoid adverse effects of anti-VEGF-based therapeutics.

Results

Impact of Anti-VEGF Blockades on Vasculature in Endocrine Organs.

To study the impact of VEGF-specific blockades on vasculatures in various healthy tissues, we used three specific anti-VEGF agents known to block VEGF-induced biological activities: a rabbit anti-mouse neutralizing monoclonal antibody (BD0801) (22); a rat anti-mouse VEGFR-1 neutralizing monoclonal antibody (MF-1) (23–25); and a rat anti-mouse VEGFR-2 neutralizing monoclonal antibody (DC101) (23–25). These antibodies were systemically delivered for 2 wk to healthy mice using doses known to block tumor angiogenesis (22–25). Because bevacizumab (a humanized anti-human VEGF neutralizing monoclonal antibody) is widely being used for treatment of various human cancers (2, 17–21) and ramucirumab (a humanized anti-human VEGFR-2 neutralizing monoclonal antibody) together with docetaxel-based chemotherapy is planned for a phase III trial for treatment of non-small cell lung carcinoma (26), systemic treatment with the same agents designed in our study would be clinically relevant.

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Fig. 3. Impact of anti-VEGF blockades on vasculature in female reproductive system. (A) CD31⁺ ovary microvessels (red). H&E staining was used to reveal tissue structures. Vessel areas were quantified (20 $\times$ magnification, $n = 8$ fields per group). (B) CD31⁺ uterus microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ fields per group). Data are presented as means $\pm$ SEM.

reduction of microvessel density in response to the VEGF blockade (Fig. 2 *A, B, and D*). A similar vascular regressive effect was also found in the VEGFR-2-treated groups. Intriguingly, microvessels in small intestinal villi showed marked decrease of vessel density in response to VEGF or VEGFR-2 blockade (Fig. 2*C*). However, systemic treatment with the VEGFR-1-specific blockade did not produce any obvious vascular changes (Fig. 2 *A–D*). In the ovary, systemic administration of VEGF and VEGFR-2 blockades produced significant effects on vascular regression whereas VEGFR-1 blockade did not show any vascular regressive effect. Surprisingly, treatment with the VEGFR-1 blockade increased vessel density in the ovarian tissue (Fig. 3*A*). There were no obvious structural changes upon anti-VEGF blockades as detected by hematoxylin/eosin histological staining. Similar to the ovary tissue, VEGF- and VEGFR-2-specific blockades significantly decreased vascular density in the uterus whereas VEGFR-1 increased vascular density in this tissue (Fig. 3*B*). These data demonstrate that the VEGF-VEGFR-2 signaling system is

essentially required for maintenance of a subset of vasculatures in these tissues and organs.

Vascular Changes in Kidney, Liver, Pancreas, and Other Tissues.

Among all analyzed tissues, renal cortex and glomeruli in the kidney showed significant reduction of vascular density in response to the VEGF-specific blockade (Fig. 4 *A and B*). The hepatic sinusoidal vasculature also responded to the VEGF-specific blockade, leading to a significant reduction of microvessel density (Fig. 4*C*). In the pancreatic acini area, the anti-VEGF treatment resulted in significant decrease of vessel numbers (Fig. 4*D*). Consistent with these findings, systemic delivery of the VEGFR-2 blockade produced a similar vascular regressive phenotype in these tissues whereas anti-VEGFR-1 blockade did not affect the vessel density (Fig. 4 *A–D*). We have also examined vasculatures in a number of other tissues including different regions of the brain, retina, thymus, myocardium, skeletal muscles, and bone marrow. In general, no significant changes were observed in these tissues and organs in response to the three VEGF blockades, with the exception of

Fig. 4. Impact of anti-VEGF blockades on vasculature in kidney, liver, and pancreas. (A) CD31⁺ renal cortex microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ fields per group). (B) CD31⁺ glomerulus microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ fields per group). (C) CD31⁺ liver microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ fields per group). (D) Endomucin⁺ pancreatic acini microvessels were detected in paraffin-embedded tissues (red). H&E staining was used to reveal tissue structures. Vessel numbers were quantified (20 $\times$ magnification, $n = 8$ fields per group). Data are presented as means $\pm$ SEM.

significant reduction of vascular density in thymus in response to VEGF and VEGFR-2 blockades (Figs. S1–S6).

Reversible Vascular Density and Architecture Recovery. Consistent with anti-VEGF-induced vascular regression, a substantial number of endothelial cells in anti-VEGF-treated thyroid vessels underwent cellular apoptosis with expression of the activated caspase-3 in endomucin⁺ endothelial structures (Fig. 5A). In fact, a more than sevenfold increase of apoptotic endothelial cell percentage was detected in the anti-VEGF-treated group relative to the untreated control group (Fig. 5A). Despite the existence of a substantial number of apoptotic endothelial cells in anti-VEGF-treated samples, thyroid follicular and other nonendothelial cells rarely became apoptotic during the 2-wk treatment period. In concordance with vascular reduction, anti-VEGF-treated thyroid tissue exhibited significant tissue hypoxia (Fig. 5G). Vascular blockade also induced thyroid vessel regression in a dose-dependent manner without altering vessel diameters (Fig. 5D). To study the anti-VEGF-induced vascular rarefaction, we performed an “on–off” experiment in which anti-VEGF drugs were delivered to healthy mice for 2 wk, followed by analysis of the thyroid vasculature at different time points (Fig. 5B). Notably, anti-VEGF-induced thyroid vascular regression was nearly completely recovered after 2-wk cessation of VEGF blockade, and vascular density and

architecture returned to the same levels seen in the untreated animals (Fig. 5C). However, we did not observe a “rebound” effect. These data demonstrate that anti-VEGF-induced vascular rarefaction is completely reversible after discontinuation of anti-VEGF treatment.

Anti-VEGF-Induced Functional Impacts in Thyroid Gland. Anti-VEGF-induced thyroid vessel regression promoted us to study the function impact of VEGF blockade. First, we measured thyroid tissue blood perfusion in anti-VEGF-treated and nontreated groups. In agreement with vascular density reduction, anti-VEGF-treated thyroid exhibited marked reduction of blood perfusion as measured using fluorescein-labeled 2,000-kDa dextran (Fig. 5E). However, blood perfusion in each individual vessel in anti-VEGF-treated and nontreated groups was not altered (Fig. 5E). Blood vessels in endocrine organs are known to contain fenestrated endothelium, and vascular fenestrations are crucial for maintenance of endocrine organ functions (28, 29). Furthermore, VEGF is the crucial factor for induction and maintenance of vascular fenestrations in endocrine organs. We next investigated the impact of anti-VEGF treatment on alteration of vascular fenestrations in thyroid gland. As expected, thyroid gland contained highly fenestrated microvasculatures with approximately more than 2 fenestrae per μm (Fig. 5H). Remarkably, VEGF blockade virtually completely suppressed

Fig. 5. Endothelial cell apoptosis, cessation-induced vascular recovery, time course, tissue hypoxia, vesicular fenestration, and thyroid functional alterations in response to anti-VEGF treatment. (A) At 2 wk after treatment with VEGF blockade, paraffin-embedded thyroid tissue sections were triple immunostained with an anti-caspase-3 antibody (green), an anti-endomucin antibody (red), and DAPI (blue). Arrows point to endothelial apoptotic cells. Caspase-3⁺ apoptotic endothelial cells were quantified ($n = 8$ per group). (B) Schematic diagram showing the treatment regimen and cessation of anti-VEGF therapy. On, on drug; Off, off drug. (C) Thyroid vascular alterations in response to VEGF blockade and withdrawal of treatment at various time points. CD31⁺ microvessels in thyroid tissues of anti-VEGF-treated and nontreated groups were quantified ($n = 8$ fields per group). (D) Thyroid vascular alterations in response to various doses of VEGF blockade and quantification of vascular CD31⁺ density and the average of vascular diameter ($n = 8$ fields per group). (E) Vascular perfusions were double immunostained with CD31 (red) and a lysinated fluorescein-labeled dextran (2,000 kDa; green). Total tissue and individual vessel perfusion were quantified ($n = 8$ fields per group). (F) Serum levels of thyroid hormone free T3 and free T4 as measured using an ELISA method ($n = 3$ samples per group). (G) Quantification of tissue hypoxia in response to anti-VEGF treatment ($n = 3$ samples per group). (H) Thyroid vascular fenestrations in anti-VEGF-treated and nontreated tissue samples and numbers of fenestrae and caveolae were quantified ($n = 8$ samples per group). M, matrix; P, perivascular cell; L, lumen. Arrows point to caveolae, and arrowheads indicate fenestrae. Data are presented as means $\pm$ SEM.

the formation of endothelial fenestrations in thyroid vessels (Fig. 5H), suggesting that VEGF in thyroid acts as a homeostatic factor for maintenance of vascular fenestrations. Interestingly, concomitant with reduction of vascular fenestrations, the number of endothelial caveolae was significantly increased in anti-VEGF-treated thyroid tissues relative to the vehicle-treated group (Fig. 5H). To further study the functional impact of anti-VEGF treatment in modulation of thyroid gland functions, mice were treated with VEGF blockade for a prolonged period of 4 wk. Importantly, the circulating level of the predominant thyroid hormone free thyroxine (T₄), but not the minimal isoform of free triiodothyronine (T₃), was significantly decreased in the VEGF blockade-treated mice compared with that of the vehicle-treated group (Fig. 5F). These findings provide compelling evidence of the endocrine functional impairment of thyroid gland after prolonged treatment with VEGF blockade.

Discussion

Since the US Food and Drug Administration (FDA) approval of the first antiangiogenic drug, bevacizumab, for treatment of metastatic colorectal cancer in 2004 (2), this anti-VEGF drug has been widely used for treatment of various human cancers, including ovarian, breast, lung, glioblastoma, and renal cell carcinoma (RCC) (17–21, 30, 31). In general, addition of bevacizumab to the standard chemotherapy significantly improves beneficial effects in cancer patients although the bevacizumab-related overall survival benefits remain modest (32). Bevacizumab in combination with chemotherapy has become the first-line standard care for treatment of several cancer types, particularly for renal cell carcinoma, non-small cell lung carcinoma, colorectal cancer, and gastrointestinal stromal tumors (19–21, 33). Additionally, ramucirumab and aflibercept, as VEGFR-2-specific and VEGF trap, respectively, are under consideration or already approved for FDA approval for oncology use (34–36). In fact, several FDA-approved TKIs that block VEGFRs, such as sunitinib, sorafenib, and pazopanib, are also commonly used antiangiogenic drugs in the clinic for treatment of human patients with RCC and other cancer types (37–39). Clinical experiences with bevacizumab and other anti-VEGF agents show that these drugs produce a range of adverse effects commonly seen in cancer patients, including hypertension, renal vascular injury often manifested by proteinuria and thrombotic microangiopathy, gastrointestinal perforation, and congestive heart failure (40–42). The anti-VEGF agent-induced broad adverse effects demonstrate that these drugs have a broad impact on vasculatures in multiple healthy tissues and organs. Although this important issue has been known for a long time, there are limited data and knowledge on how these drugs affect healthy vasculatures in various tissues. In the present study, we chose to study the systemic impact of VEGF-specific inhibitors on mice to recapitulate the clinical situation.

Among all analyzed tissues and organs, the most affected vasculatures in response to mouse VEGF and VEGFR-2 blockades have been observed in endocrine organs, particularly in thyroid. It is known that anti-VEGF agents could trigger hypothyroidism (43), suggesting that they potentially target vasculatures in the thyroid gland in human patients. We have also observed marked vascular changes in pancreatic islets where insulin-producing β -cells are located. The clinical significance of the anti-VEGF-induced vascular changes in pancreatic islets remains unknown. Similarly, clinical relevance of the anti-VEGF-induced vascular changes in adrenal cortex needs to be established. Based on our findings, we can reasonably conclude that VEGF is essentially required for maintenance of vascular homeostasis in certain endocrine organs. In the established vasculature in endocrine organs, VEGF, together with several other vascular factors, may display two main functions: maintenance of endothelial cell survivals and vascular fenestrations. We further provide evidence

showing that anti-VEGF-induced thyroid endothelial apoptosis as a potential mechanism of vascular regression, indicating that VEGF is an essential survival factor of vasculatures in endocrine glands. In the same line of this view, treatment with VEGF blockade almost completely blocked vascular fenestrations in thyroid vessels. These findings reconcile with the known vascular survival and permeability functions of VEGF (9, 14). Another interesting aspect of our study is that anti-VEGF-2, but not anti-VEGF-1, produced similar effects as VEGF blockade, demonstrating that VEGFR-2 is the primary functional receptor that transduces VEGF-mediated vascular homeostasis. Inversely, in some tissues, VEGFR-1 blockade produced a trend or significant effect to increase rather than to decrease vascular density in some tissues, suggesting that VEGFR-1 negatively regulates VEGF functions. In fact, such a negative function of VEGFR-1 in regulation of angiogenesis and homeostasis has been thoroughly discussed elsewhere (9).

Despite induction of endothelial apoptosis, cessation of VEGF blockade treatment resulted in a rapid and reversible recovery of vascular density and architecture of the thyroid vasculature, suggesting that regressed vessels can be regenerated. Although the detailed mechanism underlying vascular regeneration warrants further investigation, there was a similar regenerative mechanism of anti-VEGF-treated tumor vessels, in which angiogenic vessels grow along the basement sheet mainly made by collagen IV (44). Another interesting point is that withdrawal of VEGF blockade takes a relatively short time (approximately 2 wk) to fully recover the regressed vasculature despite the long half-life of the antibody. One possible explanation is that anti-VEGF treatment itself markedly increases VEGF expression levels in various tissues as seen in non-tumor-bearing mice (45). In supporting this view, anti-VEGF-treated tissues such as thyroid show an increased level of hypoxia, a known factor that markedly up-regulates VEGF expression levels (46, 47). High levels of VEGF rapidly neutralize the unbound free antibody molecules, resulting in defective neutralization of the antibody to VEGF. Within a relatively short period of anti-VEGF treatment, thyroid gland and perhaps other endocrine organs, exhibits extremely high capacity of compensatory functional recovery in response to vascular regression, leading to no obvious functional changes. However, prolonged treatment and persistent exposure to VEGF blockade resulted in low-level production of endocrine hormones such as thyroxine. Functional impairment of thyroid functions by chronic exposure to VEGF blockade could result from two vascular defects: vascular density reduction and elimination of vascular fenestrations.

Anti-VEGF-induced vascular changes in glomeruli may, in part, explain this type of side effect in human cancer patients (48, 49). Our data also show that VEGF-dependent vascular plasticity occurs in a tissue- and organ-specific manner. Of note, endocrine abnormalities seem to be uncommon during clinical practice of antiangiogenic drugs (50). Vasculatures in several other organs and tissues such as myocardium, brain, and skeletal muscles remain unaffected despite vascular exposure to the same dose of anti-VEGF drugs.

Taken together, our preclinical findings with systemic delivery of anti-VEGF-specific drugs in mice are in general concordance with clinically manifested adverse effects caused by these drugs in human cancer patients. Thus, our data provide a structural basis of broad targets in healthy tissues and also reveal the physiological functions of VEGF in maintenance of vascular homeostasis in various tissues and organs. Understanding the broad impacts of these anti-VEGF-specific drugs may help us to minimize or even to avoid side effects in future clinical practice.

Methods

All animal studies were approved by the Northern Stockholm Experimental Animal Ethical Committee (Stockholm, Sweden). Statistical analyses were performed using the standard two-tailed Student *t* test. Details are provided in *SI Methods*.

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Supporting Information

Yang et al. 10.1073/pnas.1301331110

SI Methods

Mice. Female 8- to 12-wk-old C57BL/6 mice were bred, acclimated, and caged in a group of six or fewer mice per cage at the animal facility of the Department of Microbiology, Tumor and Cell Biology, Karolinska Institute. Mice were killed by a lethal dose of CO₂, followed by cervical dislocation. All animal studies were approved by the Karolinska Institutet local ethical committee and the Northern Stockholm Experimental Animal Ethical Committee.

Treatment with VEGF and VEGFR Blockades. A rabbit anti-mouse VEGF-specific neutralizing antibody (BD0801) was kindly provided by the Simerce Pharmaceutical Company (Nanjing, Jiangsu, China) (1). Rat specific antibodies to mouse VEGF receptor (VEGFR)-1 (MF1) and to mouse VEGFR-2 (DC101) (2–4) were kindly provided by ImClone Systems. These antibodies were intraperitoneally (i.p.) injected twice per week into each of the mice at the dose of 5 mg/kg of the anti-VEGF antibody and 200 μ g per mouse of anti-VEGFR-1 or anti-VEGFR-2 antibodies ($n = 6$ mice per group). Tissue samples were collected at the end of the experimental duration. In the discontinuation experiment, VEGF blockade was injected i.p. two times/week into each of the mice at the dose of 5 mg/kg ($n = 6$ mice per group). Tissue samples were collected at various time points after cessation of anti-VEGF treatment, including day 0, 7, 14, and 28. For dose-dependent experiments, VEGF blockade was injected i.p. two times per week into each of the mice at the doses of 1.25, 5, and 15 mg/kg ($n = 6$ mice per group).

Tissue, Blood and Organ Collection. Mice were killed after 2 wk of antibody administration. Necropsy was performed, and various tissues and organs were removed and immediately fixed with 4% (wt/vol) Paraformaldehyde (PFA) overnight, followed by washing with PBS. A fraction of the tissues and organs was embedded with paraffin until further use. Blood samples were collected from anti-VEGF and control animals using an intracardial method (2, 3), and serum samples were kept at -20°C until further use.

Histological Studies and Whole-Mount Staining. Paraffin-embedded tissues were sectioned at a thickness of 5 μ m and stained with H&E as described (5). Whole-mount staining was performed according to our published methods (6–11). Briefly, small pieces of tissues were cut into thin slices and fixed in 4% (wt/vol) PFA overnight, followed by treatment with proteinase K (20 μ g/mL). Tissues were incubated with primary antibodies overnight at 4°C , followed by staining with secondary antibodies for 2 h at room temperature. Necessary washes with PBS were performed in between or after staining. Slides were mounted and examined under a confocal microscope (Zeiss Confocal LSM510 Microscope). By scanning 10 thin sections at 4- to 5- μ m distances of each sample, three-dimensional images of each tissue sample were projected. Quantitative analyses from at least eight different tissue sections were performed using Adobe Photoshop CS software program.

Immunofluorescence Staining. Paraffin-embedded slides were prepared at 5 μ m and baked at 60°C overnight. Antigen retrieval was achieved with an unmasking solution (Vector Labs; H3300). Bone samples were preheated for 2.5 min in a microwave oven at the maximal power and then heated for 5.5 min at 20% power. For soft samples, prolonged heating was used. Samples were

chilled to room temperature, followed by washing twice with PBS for 5 min. Samples were treated with blocking buffer [4% (vol/vol) goat serum in PBS] at room temperature for 30 min. A rat anti-mouse endomucin (eBioscience; cat. no. 14-5851-85, clone eBioV.7C7) antibody (1:200 dilution in blocking buffer) was used for incubation at 4°C overnight. A secondary antibody (goat anti-rat Alexa 555; Invitrogen; cat. no. A21434; 1:400 dilution) in the blocking buffer was incubated at room temperature for 45 min. For apoptosis assay, a rabbit anti-mouse cleaved caspase-3 antibody (Cell Signaling; cat. no. 9661; 1:200 dilution in a blocking buffer) and a secondary antibody (donkey anti-rabbit Alexa 488; Invitrogen; cat. no. A21206; 1:400 dilution) were used. Slides were mounted with Vectashield (Vector Laboratories; cat. no. H-1000) and kept in -20°C .

Vascular Perfusion Assay. Anti-VEGF-treated and nontreated mice were anesthetized and were injected into the tail vein with a lysinated fluorescein-labeled dextran (2,000 kDa; 100 μ L per mouse; Invitrogen) (12). After 10 min, animals were killed, and tissues and organs were dissected and immediately fixed with 4% (wt/vol) PFA at 4°C . Thyroids were carefully dissected, whole-mount stained, and examined by confocal microscopy.

Western Blot. After 2-wk anti-VEGF treatment, mice were anesthetized and i.v. injected with 1.5 mg pimonidazole (Hypoxyprobe). Mice were killed 15 min later, and thyroid was resected and immediately lysed. Total protein samples were collected. Fifteen micrograms of total protein was loaded into each channel and separated using a 10% (wt/vol) SDS/PAGE gel and then transferred onto a nitrocellulose membrane. Membranes were probed overnight at 4°C with a mouse anti-pimonidazole monoclonal antibody (clone 4.3.11.3, Hypoxyprobe) and diluted in PBS with 5% (wt/vol) BSA containing 0.1% Tween 20, as recommended by the manufacturer. The membrane was incubated for 1 h with a donkey anti-mouse IgG antibody (IRDye 680RD; LI-COR; 1:15000). Protein bands were visualized and quantified using ODYSSEY CLx (LI-COR) at 700 nm wavelength.

ELISA. Serum levels of mouse free triiodothyronine (T3) and free thyroxine (T4) were measured and quantified by using an ELISA method according to the manufacturer's instructions (cat. nos. MBS705057 and MBS162146; Mybiosource).

Electron Microscopy. After 2-wk anti-VEGF antibody treatment, animals were killed and immediately fixed with 2.5% (vol/vol) glutaraldehyde plus 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) by vascular perfusion. Thyroids were dissected and fixed with a fresh fixative. Electron microscopy examination and image captures were obtained as previously described (13, 14). Ultrathin tissue sections in 40–50 nm were prepared and examined in a Tecnai 12 Spirit Bio TWIN transmission electron microscope (Fei Company) at 100 kV. Digital images were taken by using a Veleta camera (Olympus Soft Imaging Solutions).

Statistical Analysis. For quantitative analysis, randomized micrographs from at least eight different fields were used. Six animals were recruited to each group, and each experiment was repeated three times. The Adobe Photoshop CS4 software program was used with a color range tool and a count tool to detect positive areas or numbers. Statistical analyses were performed using the standard two-tailed Student t test, and values of $P < 0.05$, $P < 0.01$, and $P < 0.001$ were deemed statistically significant.

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Fig. S1. Impact of anti-VEGF antibody treatment on vasculature in brain. (A) CD31⁺ olfactory bulb microvessels (red). Vessel areas were quantified (20× magnification, $n = 8$ per group). (B) CD31⁺ cerebral cortex microvessels (red). Vessel areas were quantified (20× magnification, $n = 8$ per group). (C) CD31⁺ hypothalamus microvessels (red). Vessel areas were quantified (20× magnification, $n = 8$ per group). (D) CD31⁺ cerebellum microvessels (red). Vessel areas were quantified (20× magnification, $n = 8$ per group). (E) CD31⁺ medulla oblongata microvessels (red). Vessel areas were quantified (20× magnification, $n = 8$ per group). Data are presented as means $\pm$ SEM.

Fig. S2. Impact of anti-VEGFR-1 and anti-VEGFR-2 antibody treatment on vasculature in brain. (A) CD31⁺ olfactory bulb microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ per group). (B) CD31⁺ cerebral cortex microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ per group). (C) CD31⁺ hypothalamus microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ per group). (D) CD31⁺ cerebellum microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ per group). (E) CD31⁺ medulla oblongata microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ per group). Data are presented as means $\pm$ SEM.

Fig. S3. Impact of anti-VEGF antibody treatment on vasculature in muscle, thymus, and bone marrow. (A) CD31⁺ myocardium microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ per group). (B) CD31⁺ intrinsic lingual muscle microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ per group). (C) CD31⁺ skeletal muscle microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ per group). (D) CD31⁺ thymus microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ per group). (E) Endomucin⁺ bone marrow microvessels were detected in paraffin-embedded (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ per group). Data are presented as means $\pm$ SEM.

Fig. S4. Impact of anti-VEGFR-1 and anti-VEGFR-2 antibody treatment on vasculature in muscle, thymus, and bone marrow. (A) CD31⁺ myocardium microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ per group). (B) CD31⁺ intrinsic lingual muscle microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ per group). (C) CD31⁺ skeletal muscle microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ per group). (D) CD31⁺ thymus microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ per group). (E) Endomucin⁺ bone marrow microvessels were detected in paraffin-embedded (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ per group). Data are presented as means $\pm$ SEM.

Fig. 55. Impact of anti-VEGF antibody treatment on vasculature in retina and adrenal medulla. (A) CD31⁺ retina microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ per group). (B) Endomucin⁺ adrenal medulla microvessels were detected in paraffin-embedded tissues (red). H&E staining was used to reveal tissue structures. Vessel numbers were quantified (20 $\times$ magnification, $n = 8$ per group). Data are presented as means $\pm$ SEM.

Fig. 56. Impact of anti-VEGFR-1 and anti-VEGFR-2 antibody treatment on vasculature in retina and adrenal medulla. (A) CD31⁺ retina microvessels (red). Vessel areas were quantified (20 $\times$ magnification, $n = 8$ per group). (B) Endomucin⁺ adrenal medulla microvessels were detected in paraffin-embedded tissues (red). H&E staining was used to reveal tissue structures. Vessel numbers were quantified (20 $\times$ magnification, $n = 8$ per group). Data are presented as means $\pm$ SEM.

PAPER IV

Opposing Effects of Circadian Clock Genes *Bmal1* and *Period2* in Regulation of VEGF-Dependent Angiogenesis in Developing Zebrafish

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SUMMARY

Molecular mechanisms underlying circadian-regulated physiological processes remain largely unknown. Here, we show that disruption of the circadian clock by both constant exposure to light and genetic manipulation of key genes in zebrafish led to impaired developmental angiogenesis. A *bmal1*-specific morpholino inhibited developmental angiogenesis in zebrafish embryos without causing obvious nonvascular phenotypes. Conversely, a *period2* morpholino accelerated angiogenic vessel growth, suggesting that *Bmal1* and *Period2* display opposing angiogenic effects. Using a promoter-reporter system consisting of various deleted *vegfr*-promoter mutants, we show that *Bmal1* directly binds to and activates the *vegfr* promoter via E-boxes. Additionally, we provide evidence that knockdown of *Bmal1* leads to impaired Notch-inhibition-induced vascular sprouting. These results shed mechanistic insight on the role of the circadian clock in regulation of developmental angiogenesis, and our findings may be reasonably extended to other types of physiological or pathological angiogenesis.

INTRODUCTION

In mammals and other vertebrates, the circadian clock plays a central role in regulation of physiological functions of almost all tissues and organs (Panda et al., 2002; Sehgal and Mignot, 2011). It is known that some crucial genes involved in regulation of cell growth, differentiation, and survival are tightly regulated by the clock genes (Hunt and Sassone-Corsi, 2007). Owing to the key importance of the circadian clock, certain transcription factors, such as *Bmal1* and *Clock*, and transcription coregulators *Period2* and *Cryptochrome*, or their homologs specialized for circadian regulation of targeted genes, are widely present in vertebrates, eukaryotic cells, and even prokaryotic cells (Schultz and Kay, 2003). As such, disruption of the circadian clock may

lead to the onset, development, and progression of certain diseases, including neuronal disorders, metabolic disorders, chronic inflammation, and even cancer (Allada, 2003). Therefore, understanding the molecular mechanisms that control the circadian clock is pivotal for prevention and treatment of these common human diseases.

Angiogenesis is an essential process for embryonic development and for maintenance of certain physiological functions in the adult (Folkman, 1995). For example, the female reproductive cycle and wound healing are dependent on angiogenesis. Under pathological conditions, uncontrolled growth of new blood vessels could lead to the onset and development of diseases. Tumor growth and metastasis are dependent on neovascularization, and targeting pathological blood vessels in tumors has been demonstrated as an effective approach for cancer therapy in human patients (Cao and Langer, 2010; Folkman, 2007; Kerbel and Folkman, 2002). Additionally, antiangiogenic therapy has produced remarkably beneficial effects on vision improvement in patients with age-related macular degeneration (Stone, 2006).

Angiogenesis is regulated by both positive and negative factors, and the expression levels of these factors can be altered under pathological conditions (Cao, 2009a, 2009b). It is generally believed that an angiogenic switch is turned on by tipping the balance of angiogenic factors and inhibitors toward domination of stimuli. Members of the vascular endothelial growth factor (VEGF) family are the most well characterized, which include VEGF (also called VEGF-A), -B, -C, -D, and placental growth factor (PlGF) (Cao, 2009a). These angiogenic factors bind to three tyrosine kinase (TK) receptors, VEGFR-1, VEGFR-2, and VEGFR-3 expressed on the surface of blood or lymphatic vessel endothelial cells to exert their biological functions (Caunt et al., 2008; Pan et al., 2007; Soker et al., 1998). It is generally believed that VEGFR-2 is the functional receptor that transduces angiogenic and vascular permeability signals in response to VEGF stimulation. In contrast to VEGFR-2, the function of VEGFR-1 remains poorly understood, and it may act as a decoy receptor for the VEGF-triggered signaling (Cao, 2009a; Eriksson et al., 2002). The Dll4-Notch signaling system has recently been reported to restrict vascular sprouting and to modulate vascular remodeling and maturation (Hellström et al., 2007; Noguera-Troise et al., 2006; Ridgway et al., 2006; Siekmann and Lawson,

2007). These functions are tightly associated with modulation of the VEGF-induced angiogenic activity. Inhibition of Dll4 or the Notch receptor signaling system leads to an increased number of vascular sprouts in tumors and other tissues (Cao et al., 2008; Noguera-Troise et al., 2006).

Despite the vast knowledge of circadian biology and angiogenesis, the role of the circadian clock in regulation of angiogenesis and vascular patterning remains poorly understood. In the present study, we use developing zebrafish embryos to demonstrate the crucial role of the circadian clock in controlling vascular network formation and patterning. Our findings may imply novel therapeutic options for the treatment of angiogenesis-dependent diseases, such as cancer and cardiovascular diseases, by targeting the circadian clock.

RESULTS

Exposure to Constant Light, but Not Constant Darkness, Impairs Vascular Development

To study the impact of the circadian clock on vascular development, *Tg(Fli1:egfp)* zebrafish (Lawson and Weinstein, 2002) embryos at the age of 1 hr postfertilization (hpf) were exposed to constant cold-light (LL, 1800 lux), constant darkness (DD), or a 12 hr light-dark (LD) cycle. Under identical conditions except the light source, LL-exposure neither affected the zebrafish viability nor caused any abnormal phenotypes (Figure 1A). Similarly, DD exposure did not result in any morphological changes of developing zebrafish. Notably, the somite length and the head angles, two widely used parameters for staging the development of zebrafish embryos (Kimmel et al., 1995), were virtually identical in LL- or LD-exposed zebrafish embryos (Figure 1B). These findings indicate that LL-exposure is well tolerated by zebrafish embryos. At 24 hpf under constant light exposure, development of intersegmental vessels (ISVs) was significantly impaired relative to those embryos exposed to LD (Figure 1C). Quantification analysis showed that the average length of ISVs in LL-exposed zebrafish embryos was significantly shorter relative to those embryos exposed to LD (Figure 1D). Similar to ISVs, exposure of zebrafish embryos to LL until 72 hpf led to significantly decreased development of subintestinal veins (SIVs, Figures 1C and 1D). In contrast to LL, DD exposure did not result in significant alterations of ISVs or SIVs as compared to LD exposure (Figures 1C and 1D). These findings demonstrate that LL exposure significantly impairs developmental angiogenesis in zebrafish.

To study the impact of LL and DD exposure in modulation of *period2*, a known clock gene to be affected by light (Vatine et al., 2009), *period2* expression levels were quantitatively analyzed using an RT-PCR method. As expected, *period2* expression under LD conditions exhibited circadian rhythmicity, being upregulated during light exposure and downregulated during the dark phase (Figures 1E and 1F). Interestingly, DD exposure led to marked regression of *period2* expression levels, which were barely detectable at the various hpf time points. Conversely, LL exposure resulted in persistent upregulation of *period2* (Figures 1E and 1F). These findings demonstrate that exposure of zebrafish embryos to LL and DD could lead to differential expression of the clock gene *period2*.

Opposing Effects of Bmal1 and Period2 on Developmental Angiogenesis

To further delineate the role of the circadian clock in regulation of developmental angiogenesis, we utilized the morpholino technology to interrupt the circadian clock in zebrafish embryos by knocking down expression of some critical circadian genes, including *bmal1*, *bmal2*, *period2*, and *clock3* (Allada, 2003). These morpholinos have previously been shown to impair pineal gland development (Appelbaum et al., 2005; Triqueneaux et al., 2004; Vatine et al., 2009; Ziv et al., 2005). As the pineal gland serves as a master circadian pacemaker, disruption of its development by these morpholinos would have a significant impact on the circadian clock as a whole. To demonstrate that these morpholinos sufficiently blocked translation of their corresponding clock gene products, zebrafish cDNAs coding for clock genes, including *bmal1*, *bmal2*, *clock3*, and *period2*, were cloned into a pCS2-C-myc vector, which allowed generation of fusion proteins with a myc-tag. The in vitro-transcribed mRNAs were injected with or without morpholinos into zebrafish embryos. As shown in Figure S1, *bmal1*, *bmal2*, *clock3*, and *period2* morpholinos effectively blocked translation of injected corresponding mRNA in zebrafish embryos as detected by an anti-myc-tag antibody. These data demonstrate that the morpholinos used in our study efficiently knocked down their corresponding clock gene products.

Interestingly, injection of the *bmal1* morpholino at the one-cell stage of developing zebrafish embryos under LD led to marked repression of ISV development compared to scrambled or mismatch *bmal1* morpholinos (Figures 2A and 2B). Downregulation of the Bmal1 function by the specific morpholino resembled the LL-induced vascular impairment (Figure 1). Surprisingly, knockdown of *Period2* under LD conditions did not suppress vascularization but unexpectedly enhanced ISV growth as compared with the mismatch morpholino control (Figures 2A and 2B). These findings suggested that Bmal1 and *Period2* might display opposing effects on embryonic vessel development under the LD physiological condition. Morpholinos specifically targeting *bmal2* and *clock3* were also tested in the same experimental setting. In contrast to *bmal1* and *period2* morpholinos, delivery of *bmal2* or *clock3* morpholinos to zebrafish embryos did not significantly affect ISV development (Figures 2A and 2B). Similarly, a combination of *bmal1* and *bmal2* morpholinos did not produce any additive effects as compared with *bmal1* morpholino alone (Figures 2A and 2B).

To gain further molecular information on the LL-induced vascular impairment in relation to circadian genes, Bmal1-knock-down zebrafish embryos were exposed to LL. Intriguingly, knockdown of Bmal1 followed by LL exposure did not produce an additive effect on the impairment of ISVs, suggesting that downregulation of Bmal1 mediates LL-induced vascular retardation (Figures 2A–2C). Conversely, knockdown of *Period2* under LL exposure completely rescued the LL-induced vascular defects on ISV development (Figures 2A–2C), validating the negative role of *Period2* in regulation of vascular development. Indeed, quantitative real-time PCR (qPCR) analysis demonstrated that *period2* was markedly upregulated at 48 and 72 hpf under LL exposure (Figure 2I). Taken together, these findings demonstrate that Bmal1 acts as a positive mediator

Figure 1. Constant Light Exposure Inhibits Developmental Angiogenesis

Tg(Fli1:EGFP)¹ transgenic zebrafish embryos exposed to LD (A–D), LL (A–D), or DD (C and D) were examined for ISV development at 24 hpf (C and D) or for SIV development at 72 hpf (C and D) using confocal microscopy.

(A) Morphology of zebrafish embryos was examined at 24 hpf and 48 hpf under LD and LL conditions.

(B) Average $\pm$ SEM somite lengths and head angles were quantified under LD and LL conditions ($n = 7$ –9/group).

(C) Zebrafish embryos were raised under LD, DD, or LL conditions from 1 hpf. Dashed lines indicate the maximal length of ISVs. Yellow dashed lines encircle the SIV. Scale bar for ISV is 50 μ m and for SIV is 100 μ m.

(D) Quantification of the average $\pm$ SEM. ISV length ($n = 52$ –54 embryos/group) of 24 hpf embryos and the SIV area of 72 hpf embryos ($n = 48$ embryos).

(E) RT-PCR analysis of average zebrafish *period2* (P2) expression $\pm$ SEM under LD, LL, and DD conditions (a pooled sample of 25 zebrafish embryos/group).

(F) Densitometric quantification of average *period2* expressions shown in (E).

Bars in (A) = 500 μ m. S, somite; NC, notochord; H, hindbrain; M, mesencephalon; E, eye. Blue lines in (A) indicate the head angle. * $p < 0.05$ and ** $p < 0.01$. NS, not significant.

See also Figure S2.

for vascular development, whereas Period2 negatively regulates angiogenesis.

Similar to LL exposure, neither *bmal1* nor *period2* morpholinos caused nonvascular defects, including changes in the somite development and the head angles (Figures 2D–2E). The vascular specific effects of *bmal1* and *period2* morpholinos were further validated using other specific organ or tissues markers. In situ hybridization showed that expression of sonic hedgehog (*shh*), a key morphogen during organogenesis and expression of

acetylated alpha tubulin, a marker for axon maturation and stability was not altered (Figure S2).

Restoration of LL- or *bmal1* Morpholino-Induced ISV Defects by *bmal1* mRNA

To further validate the role of Bmal1 and Period2 in modulation of ISV development, we performed gain-of-function experiments by injection of zebrafish *bmal1* or *period2* mRNA under various conditions. As expected, injection of *bmal1* mRNA resulted in

Figure 2. Opposing Roles of Bmal1 and Period2 in Regulation of Developmental Angiogenesis

(A) Zebrafish embryos received injections of specific morpholinos targeting Bmal1 (B1MO), Bmal2 (B2MO), Clock3 (C3MO), or Period2 (P2MO) (0.2 pmol/embryo) and were subsequently raised under LL or LD conditions. Scrambled morpholino (scrMO), five base-pair mismatch *bmal1* (B1misMO), or five base-pair mismatch *period2* (P2misMO) were used as controls. Dashed lines indicate the maximal length of ISVs. Bar = 50 μ m.

(B) Quantification of the average length $\pm$ SEM of ISVs in 24 hpf embryos under the LD condition (n = 8–48 embryos/group).

(C) Quantification of the average length $\pm$ SEM of ISVs in 24 hpf embryos under the LL condition (n = 6–37 embryos/group).

(D) Morphology of zebrafish embryos was examined at 24 hpf and 48 hpf under LD and LL conditions. Bars = 500 μ m. S, somite; NC, notochord; H, hindbrain; M, mesencephalon; E, eye.

(E) Quantification of average $\pm$ SEM somite lengths and head angles under LD and LL conditions (n = 7–17/group).

(F) Zebrafish embryos received injections of specific morpholinos targeting Bmal1 (B1MO), Period2 (P2MO), scrambled morpholino (scrMO), *bmal1* mRNA (B1mRNA), *period2* mRNA (P2mRNA), B1mRNA plus B1MO, or P2mRNA plus P2MO and were subsequently raised under LD or LL conditions. Dashed lines indicate the maximal length of ISVs. Bar = 50 μ m.

(G) Quantification of the average length $\pm$ SEM of ISVs in 24 hpf embryos under the LD condition (n = 8–27 embryos/group).

(H) Quantification of the average length $\pm$ SEM of ISVs in 24 hpf embryos under the LL condition (n = 6–28 embryos/group).

(I) qPCR of average $\pm$ SEM *period2* mRNA expression levels after 48 or 72 hpf LD or LL exposure (n = 3–4 $\times$ 20–50 embryos/group). *p < 0.05; **p < 0.01; and ***p < 0.001. NS, not significant.

See also Figures S1 and S2.

a significant increase of ISV length (Figures 2F–2H). Intriguingly, sequential injection of *bmal1* morpholino followed by *bmal1* mRNA significantly restored ISV development (Figures 2F and 2G). Similarly, delivery of *bmal1* mRNA completely rescued the LL-induced ISV defects (Figures 2F–2H). Conversely, administration of *period2* mRNA significantly antagonized the *period2* morpholino-induced vessel growth (Figures 2F and 2H). These findings demonstrate that the morpholinos used in our study were specific for *bmal1* and *period2* and further validate the vascular role of these clock genes.

VEGF Mediates Bmal1-Induced Angiogenesis

VEGF is a key angiogenic factor during embryonic development and in pathological conditions, including tumor growth and ophthalmological disorders (Ferrara et al., 2003; Nagy et al., 2007). In mice, heterozygous deletion of the *vegfr* gene leads to early embryonic lethality owing to lack of vascular and hematopoietic development, demonstrating that an optimal level of VEGF is essential to ensure appropriate development of various tissues and organs (Carmeliet et al., 1996; Ferrara et al., 1996). To study the involvement of VEGF and its receptor signaling system in mediating circadian-modulated angiogenesis, a specific *vegfr* morpholino (Nasevicius et al., 2000) was used in combination with *bmal1* or *period2* morpholinos. As expected, delivery of a high dose of *vegfr* morpholino (0.2 pmol/embryo) to zebrafish embryos resulted in virtually complete suppression of ISV growth at 24 hpf (Figure 3B), suggesting that VEGF is absolutely required for ISV development. However, injection of a low dose of *vegfr* morpholino (0.06 pmol/embryo) led to an only partial but statistically significant inhibition of ISV development (Figures 3A and 3C). Interestingly, a combination of *vegfr* and *bmal1* morpholinos additively inhibited ISV growth, whereas coinjection of *vegfr* and *period2* morpholinos did not result in an additional inhibitory effect but a slightly increased angiogenic response (Figures 3A and 3C).

Similar to the *vegfr* morpholino, addition of an orally active VEGFR tyrosine kinase inhibitor (ZN323881, TKI) (Cao et al., 2008) to the aquarium water at a low concentration resulted in partial suppression of ISV development (Figures 3A and 3C). Again, delivery of a combination of ZN323881 and the *bmal1* morpholino resulted in an additive effect on suppression of the ISV growth (Figures 3A and 3C). Conversely, a combination of ZN323881 and *period2* did not further potentiate the inhibitory activity as compared with ZN323881 alone. An independent experiment using sunitinib, another orally active VEGFR TKI, produced virtually identical results (Figure S3).

Bmal1 Targets the *vegfr* Promoter for Transcriptional Activation

To provide direct evidence that the *vegfr* gene is a direct target for Bmal1, a *vegfr* promoter-luciferase reporter system was used to study regulation of *vegfr* promoter activity by the circadian clock in zebrafish embryos. A *bmal1* promoter-luciferase reporter system was also constructed to measure alteration of the Bmal1 level by the circadian clock. We used both human and zebrafish *vegfr* promoter-luciferase in our studies. Intriguingly, an almost perfect coordination of expression patterns was observed between human VEGF and mouse Bmal1 under LD

exposure (Figure 3G), suggesting that VEGF expression levels are modulated by the circadian clock. Strikingly, the correlation between *bmal1* and *vegfr* promoter activity was disrupted under LL conditions (Figure 3G). Inversely, *bmal1* and *vegfr* promoter activity coincided and exhibited circadian rhythmicity under DD conditions (Figure 3G).

Similarly, zebrafish *vegfr* promoter activity was also coordinately regulated by the circadian clock under LD and DD conditions (Figure 3H), and LL-exposure significantly suppressed zebrafish *vegfr* promoter activity (Figure 3D). To validate these findings, we measured the endogenous levels of *bmal1* and *vegfr* mRNA in zebrafish using a qPCR method. In principle, the circadian expression pattern of *bmal1* matched that of *vegfr* under LD exposure (Figure 3I). Particularly, endogenous expression levels of zebrafish *bmal1* and *vegfr* exhibited coordinated rhythmicity even during prolonged LD exposure (Figures S4A and S4B). These findings provide compelling evidence that VEGF coincides with Bmal1 expression under LD conditions. Again, LL exposure almost completely ablated the rhythmic expression of *vegfr* and *bmal1* genes (Figure 3I), indicating that expression levels of *bmal1* and *vegfr* are tightly regulated by the circadian clock. Similar to LD conditions, zebrafish embryos under DD exposure retained the rhythmicity of *bmal1* and *vegfr* expression (Figure 3I).

As an independent proof of the intimate interaction between Bmal1 and VEGF expression, knockdown of Bmal1 led to significantly decreased levels of human and zebrafish VEGF expression (Figures 3E and 3F), demonstrating that Bmal1 is required to maintain a steady-state level of VEGF expression. These findings provide a mechanistic insight that Bmal1 might directly target the *vegfr* promoter for transcriptional regulation.

To study if Bmal1 directly targets the *vegfr* promoter, we analyzed the promoter regions of the *vegfr* genes in zebrafish, mice, and humans. In zebrafish, we identified four confirmed E-box regions that potentially bind to the Bmal1-transcription complex (Figures 3J and 3K). There were totally five such Bmal1-binding E-boxes on the promoter region of the mouse *vegfr* gene and one exist in the promoter region of the human *vegfr* gene (Figure 3J). In addition, multiple unconfirmed Bmal1-binding E-boxes exist in all three species and these E-box sites are likely to bind to Bmal1. To study if E-boxes in the zebrafish VEGF promoter are involved in Bmal1-regulated VEGF expression, a series of E-box-deleted mutants of the promoter was generated (Figure 3K). Deletion of E-box 1 resulted in significant reduction of the *zvegfr* promoter activity (Figures 3K and 3L). Similarly, deletion of the cluster E-boxes 2–4 virtually completely abolished the *zvegfr* promoter activity (Figures 3K and 3L). These findings demonstrate that E-boxes in the *zvegfr* promoter are essentially required for transcriptional regulation of VEGF expression. We next performed a chromatin immunoprecipitation assay in which an anti-mouse Bmal1 antibody was used to precipitate Bmal1 protein using a mouse cell line. The crosslinked DNA-protein complexes were then disassociated and PCR amplified using several pairs of specific primers covering various regions of the *vegfr* promoter. An E-box-less *vegfr* fragment was used as a negative control, and a fragment containing the first two E-boxes in the *period1* gene was used as a positive control as previously described (Jung et al., 2003). As expected,

fragments containing E-boxes 1–3 and 4–5 in the *vegfr* promoter region showed direct binding to Bmal1 (Figure 3M), whereas the E-box-less fragment did not show significant binding activity. Taken together, these results provide compelling evidence that Bmal1 directly binds to the *vegfr* promoter via E-boxes.

Relation of Circadian and Notch Signaling in Regulation of Vessel Development

To study the involvement of the Notch signaling system in modulation of the circadian clock-VEGF axis, three independent approaches were used to block the Notch pathway. First, a γ -secretase inhibitor (DAPT) that inhibits the Notch signaling system was used. As expected, addition of DAPT to the aquarium water resulted in the formation of high-density vascular networks and a high number of vascular branches from ISVs (Figures 4A and 4B). Second, we designed a morpholino specifically targeting recombining binding protein suppressor of hairless (*rbpsuh*) (Siekmann and Lawson, 2007) that mediates the Notch signaling. Similar to DAPT, delivery of the *rbpsuh* morpholino also led to the formation of a disorganized ISV network that consisted of a high density of microvessels that grew in widely different directions (Figures 4A and 4B). Finally, a genetic mutant strain of zebrafish that lacks functional mind-bomb, a ubiquitin ligase involved in mediating the Notch signaling (Haddon et al., 1998), was used. Again, a high degree of ISV malformation and excessive vascular branches/sprouts existed in the mind-bomb mutant zebrafish (Figures 4A and 4B). These findings provide compelling evidence that inhibition of the Notch signaling system leads to the formation of a high density of disorganized vascular networks in the ISV region.

Interestingly, delivery of the *bmal1* morpholino to zebrafish embryos markedly reduced the Notch inhibition-induced vascular malformation and excessive vascular sprouts (Figures 4A and 4B). Inversely, the *period2* morpholino did not significantly affect the Notch inhibition-induced vascular phenotype

(Figures 4A and 4B). Administration of the *vegfr* morpholino at a low dose to zebrafish embryos partly prevented the Notch inhibition-induced vascular phenotypes (Figures 4A and 4B), suggesting that VEGF mediates the Notch-induced vascular changes. As described above, expression levels of VEGF and Bmal1 were downregulated under the LL condition. Under LL exposure, the Notch inhibition-induced vascular malformation was partially prevented (Figures 4C and 4D). On the contrary, knockdown of *Period2* under LL further potentiated the Notch inhibition-induced vascular phenotype (Figures 4C and 4D). These findings suggest that the circadian clock-regulated VEGF expression levels act as a driving force for the Notch-induced vascular changes.

DISCUSSION

The circadian clock controls growth, migration, differentiation, and survival of many cell types and is involved in regulation of physiological and pathological processes, such as embryonic and disease development. Angiogenesis is required for embryonic development and excessive or defective blood vessel growth has been linked to various human diseases (Cao, 2009a; Cao et al., 2011). However, the role of the circadian clock in regulation of angiogenesis under physiological and pathological conditions remains poorly understood. Using zebrafish as an *in vivo* model, we, in the present study, provide compelling evidence that developmental angiogenesis is tightly controlled by the circadian clock. At the molecular level, we showed that Bmal1 and *Period2* displayed opposing effects on vascular development via regulation of VEGF expression. The circadian clock-regulated VEGF levels crosstalk with the Notch signaling system in controlling the extent of vessel sprouts and functions. Although the study was performed in zebrafish, our data may be reasonably extended to mammals, including humans because the clock genes are highly conserved among these species.

Figure 3. Circadian Clock Regulates Expression Levels of VEGF

- (A) Zebrafish embryos were injected with scrambled (scrMO) morpholino (A) or a low dose of the *vegfr* morpholino (VEGFMO, 0.06 pmol/embryo) alone or in combinations with *bmal1* (B1MO) or *period2* (P2MO) morpholinos (0.2 pmol/embryo). Some embryos were treated with either DMSO vehicle or a low dose of VEGFR TKI (0.3 μ M ZN323881) alone or in combinations with *bmal1*, *period2*, or scrambled morpholinos (0.2 pmol/embryo). The embryos were subsequently raised under the LD condition.
- (B) High doses of the *vegfr* morpholino (0.2 pmol/embryo) or ZN323881 (1.0 μ M) were also delivered to zebrafish embryos. Dashed lines in (A and B) indicate the maximal length of ISVs in various groups. Bars = 50 μ m. Quantification of the average ISV length $\pm$ SEM in 24 hpf embryos (n = 12–68 embryos/group).
- (C) Quantification of the average ISV length $\pm$ SEM in 24 hpf embryos from the experiment shown in (A) (average n = 36 embryos/group).
- (D) Zebrafish *vegfr* promoter activity as measured by average luciferase activity $\pm$ SEM in zebrafish embryos exposed to LD or LL (n = 20 embryos/group).
- (E) Zebrafish *vegfr* promoter activity in scrambled or *bmal1* morpholino-treated zebrafish embryos as measured by average luciferase activity $\pm$ SEM (n = 20 embryos/group).
- (F) Human *vegfr* promoter activity in scrambled or *bmal1* morpholino-treated zebrafish embryos as measured by average luciferase activity $\pm$ SEM (n = 20 embryos/group).
- (G–H) Values of average relative luciferase activity $\pm$ SEM in embryos exposed to LD, LL, or DD. Black line: Luciferase activity in zebrafish embryos injected with human *vegfr* promoter-luciferase (G) or zebrafish *vegfr* promoter-luciferase (H). Red lines in G represent luciferase activity in zebrafish embryos injected with a mouse *bmal1* promoter-luciferase reporter plasmid.
- (I) qPCR analysis of average $\pm$ SEM endogenous levels of *bmal1* (red line) and *vegfr* (black line) under LD, LL, and DD conditions.
- (J) Sequence analysis of human, mouse, and zebrafish *vegfr* promoter regions that contain E-boxes that bind to Bmal1. Red boxes represent the known Bmal1 binding E-boxes, and white boxes represent unknown binding E-boxes.
- (K) Schematic diagram showing various deleted mutants of the zebrafish *vegfr* promoter region. ptr, promoter.
- (L) Average luciferase activity $\pm$ SEM in embryos injected with various deleted mutants of the zebrafish *vegfr* promoter-luciferase and subsequently raised under LD exposure (n = 20 embryos/group).
- (M) ChIP assay of average Bmal1 binding $\pm$ SEM to the zebrafish *vegfr* promoter. EB, E-box. *p < 0.05; **p < 0.01; and ***p < 0.001. NS, not significant. See also Figures S3 and S4.

Figure 4. Involvement of the Notch Signaling Pathway in Circadian Clock-Regulated Vascular Sprouting
(A) Zebrafish embryos received *bmal1* (B1MO, 0.2 pmol/embryo), *period2* (P2MO, 0.2 pmol/embryo) or *vegfr* (VEGFMO, 0.1 pmol/embryo) morpholinos, either alone or in combination with DAPT (100 μ M), *rbpsuh* morpholino (RbpsuhMO, 0.1 pmol/embryo), or in the mind-bomb background. The embryos were subsequently raised under the LD condition. Arrows point to ISV branches in each group. Scale bar is 100 μ m.
(B) Quantification of the average $\pm$ SEM. ISV density or number of branches/segment of 72 hpf embryos in (A) (average $n = 23$ embryos/group).
(C) Zebrafish embryos received *period2* (P2MO, 0.2 pmol/embryo) alone or in combination with DAPT (100 μ M), *rbpsuh* morpholino (RbpsuhMO, 0.1 pmol/embryo), or in the mind-bomb background. The embryos were subsequently raised under LD or LL conditions. Arrows point to ISV branches in each group. Scale bar is 100 μ m.
(D) Quantification of the average $\pm$ SEM. ISV density or number of branches/segment of 72 hpf embryos in (C) (average $n = 23$ embryos/group). * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. NS, not significant.

During embryogenesis, vascular development is tightly regulated by various positive and negative regulators. Optimal levels of these vascular modulators are essential to ensure the formation of healthy and functional vascular networks that perfuse sufficient O_2 and nutrients for tissue and organ development (Carmeliet et al., 1996; Ferrara et al., 1996; Gale et al., 2004). Inadequate expression levels of key angiogenic factors might lead to the malformation of blood vessels, which eventually terminates development of the entire embryo. For example, genetic deletion of only one allele of the *vegfr* gene or the *dlla4* gene in mice (haploinsufficiency) leads to early embryonic death owing to vascular defects (Carmeliet et al., 1996; Ferrara et al., 1996; Gale et al., 2004). These studies suggest that an optimal level of VEGF is absolutely required for proper vascular development, navigation of vessel outgrowth, and maintenance of vascular functions. The VEGF expression level can be modulated

by multiple factors in tissue microenvironments. For example, tissue hypoxia potently upregulates VEGF expression (Boutin et al., 2008; Makino et al., 2001). In this study, we have shown that the circadian clock directly modulates VEGF expression levels via transcriptional regulation during embryonic development. First, we show that VEGF expression in developing embryos exhibits a clear circadian rhythm. Second, the VEGF expression pattern almost completely matches the expression pattern of *Bmal1*, which is an essential component of the circadian clock-induced gene expression program. Third, we provide molecular evidence that the *vegfr* promoter contains several *Bmal1* binding E-boxes and that *Bmal1* directly binds to the *vegfr* promoter via these E-boxes. Finally, we showed that knockdown of *Bmal1* directly reduced the *vegfr* promoter activity using a luciferase reporter system in whole zebrafish embryos. These data provide convincing evidence that the

circadian clock directly regulates VEGF expression and vascular development.

One of our intriguing findings is that LL and DD exposure leads to differential regulation of some clock genes, although both conditions are known to disrupt the circadian clock. This interesting finding is discussed in more detail in the [Extended Discussion](#).

The implication of our findings might be extended to regulation of angiogenesis under pathological conditions. For example, it is known that disruption of the circadian clock may increase cancer incidence (Hrushesky et al., 1999; Stevens et al., 2011). It is plausible that the circadian clock-regulated VEGF expression and vascular development might significantly contribute to tumor development. This possibility warrants further investigation. Another interesting finding of our study is the relation between the circadian clock-modulated VEGF levels and the Notch signaling-induced vascular remodeling. Notch signaling restricts vascular sprouts by limiting endothelial tip cell formation and maintaining arterial identity, which is dependent on its relation to VEGF (Noguera-Troise et al., 2006; Ridgway et al., 2006). VEGF is the driving force of tip cell formation, vascular sprouting and the formation of primitive vascular networks. We showed that downregulation of VEGF expression by interruption of the circadian clock or knocking down Bmal1 leads to an abridged effect of Notch inhibition-induced vascular malformation. Interestingly, inhibition of NADPH oxidases is associated with downregulation of Dll4 and Notch signaling (Bhandarkar et al., 2009). This signaling system in relation to the circadian clock warrants further study.

Despite vascular defects under circadian clock-disrupted conditions, zebrafish development was not significantly affected. Unlike mammals, zebrafish embryos undergo persistent development for approximately 1 week in the absence of vasculature or circulation (Stainier et al., 1995). These avascular zebrafish embryos are dependent on free diffusion of oxygen and nutrients. Also, compensatory mechanisms may exist in zebrafish because of the many clock gene homologs. For example, three Bmal, three Clock, four Period, and six Cryptochrome homologs have been reported to exist in zebrafish (Wang, 2008a, 2008b, 2009), and they may have redundant and distinct functions. In the present study, we provide an example of opposing vascular functions of Bmal1 and Period2. Unlike Bmal1 and Period2, knockdown of Bmal2 or Clock3 did not cause any obvious phenotypes. Additionally, several other clock gene products, including Clock1, Clock2, and Period3, were investigated using the morpholino approach. The phenotypes of Clock1-, Clock2-, and Period3-deficient zebrafish embryos are under investigation.

Taken together, our data provide mechanistic insight on circadian clock-regulated vascular development. Maintenance of the normal circadian clock would be essential to ensure proper development of vasculatures in the embryos and probably in other physiological and pathological conditions.

EXPERIMENTAL PROCEDURES

Zebrafish Strains and Husbandry

Zebrafish of the *Tg(fli1:EGFP)^{y1}* and *Tg(Mib^{ts2b/ta52b}:fli1:EGFP)^{y1}* strains (ZF1N, Eugene, OR, USA) were used in this study, as previously described

(Cao et al., 2010; Rouhi et al., 2010). All experiments were performed in accordance with ethical permissions granted by the North Stockholm Experimental Animal Ethical Committee, Sweden.

Morpholino Treatment

All morpholinos used in this study were purchased from Gene Tools (Philomath, OR, USA).

Assessment of Morpholino Efficiency

Amplified fragments of the *bmal1a*, *bmal2*, *period2*, and *clock3* open reading frames were cloned into the pCS2-C-Myc vector to generate C-terminal myc-tag fusion constructs. Myc-fusion proteins were detected in the presence or absence of morpholino by immunohistochemistry with a 9E10 monoclonal anti-myc antibody (Sigma-Aldrich, St. Louis, MO, USA).

RT-PCR

Total RNA was reverse-transcribed using a RevertAid H minus First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA). RT-PCR was performed using DreamTaq Green PCR Master Mix (Thermo Scientific, Waltham, MA, USA). See the [Extended Experimental Procedures](#) for a list of primers used in this study. Bands were quantified by the densitometry function of the Quantity One software.

Zebrafish VEGF-Luciferase Construction and Promoter Deletion

Promoter fragments of different sizes for the zebrafish *vegfab* gene were generated by PCR from zebrafish genomic DNA and cloned into the pGL3-Basic vector (Promega, Madison, WI, USA).

Promoter Activity Assay

Zebrafish embryos were injected with human *vegfr* promoter-luciferase, mouse *bmal1* promoter-luciferase, or zebrafish *vegfab* promoter-luciferase, followed by immediate transfer to LD, LL, or DD conditions. The dual luciferase kit (Vector Laboratories, Burlingame, CA, USA) was used for detection of luciferase activity.

Quantitative Real-Time PCR

RNA purification was performed from whole embryos using a GeneJET RNA purification kit (ThermoFisher Scientific). RNA levels of zebrafish genes were detected by qPCR, as described previously (Xue et al., 2008).

Generation of Capped mRNA

Capped mRNA was prepared using the mMessage/Machine Kit (Ambion, Austin, TX, USA). The full-length EST clone of Bmal1 (IMAGE 2643691) was subcloned into pCS2+.

Chromatin Immunoprecipitation Assay

Mouse fibroblast cells were used for the EZ-ChIP chromatin immunoprecipitation assay (Millipore, Billerica, MA, USA). A rabbit anti-Bmal1 antibody (2.5 µg per sample; Abcam, Cambridge, UK) and a nonimmune rabbit IgG (2.5 µg per sample; Millipore) were used for immunoprecipitation. The purified DNA was used for qPCR analysis. See the [Extended Experimental Procedures](#) for a list of primers used.

Zebrafish Histology

Zebrafish immunohistochemistry and in situ hybridization was performed according to published protocols (Bräutigam et al., 2011) using antiacetylated tubulin antibodies (Sigma-Aldrich cat no. T-6793; 1:1,000 dilution) and a cDNA EST clone coding for zebrafish sonic hedgehog (Imagines, SHH: IMAGE ID 9037826). Stained zebrafish were analyzed under a Leica MZ16 microscope equipped with a Leica DFC300FX camera.

Microscopy and Imaging Analysis

Zebrafish embryos were whole-mounted in VectaShield (Vector) and examined under a confocal microscope (Nikon D-Eclipse C1), as previously described (Dahl Ejby Jensen et al., 2009; Lee et al., 2009).

Statistical Analysis

Statistical analyses were done using the standard two-tailed Student t test, and * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were considered statistically significant. The n values indicate the number of individual embryos analyzed. Data were presented as means $\pm$ SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes an Extended Discussion, Extended Experimental Procedures, and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2012.07.005>.

LICENSING INFORMATION

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EXTENDED DISCUSSION

Period2 is markedly downregulated under DD conditions and downregulation of this negative regulator of angiogenesis may in part explain why DD exposure did not significantly inhibit developmental angiogenesis in zebrafish embryos. In contrast to LL exposure, zebrafish VEGF levels retained rhythmicity under DD exposure during our experimental periods, although prolonged DD exposure may lead to more severe disruption of the circadian rhythm (Cahill, 2002; Carr et al., 2006; Tamai et al., 2005, 2007). These findings further support differential regulation of clock genes and VEGF expression under LL and DD conditions.

Other independent studies also support the fact that LL and DD conditions differentially regulate Period2 expression (Pando et al., 2001; Vatine et al., 2009). Although our findings demonstrated regulation of developmental angiogenesis by clock genes, we cannot completely exclude the possibility of a circadian clock-independent mechanism underlying the Bmal1-modulated angiogenesis. This possibility warrant further mechanistic studies using zebrafish and perhaps mammalian genetic models.

EXTENDED EXPERIMENTAL PROCEDURES

Zebrafish Strains and Husbandry

Zebrafish were kept at Södertörns University, Huddinge, Sweden; or at the Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden. All embryos were produced by natural matings, and maintained in standard 0.3x Danieau's or E3 embryo medium at 28.5°C. All experiments were performed in accordance with ethical permissions granted by the North Stockholm Experimental Animal Ethical Committee, Sweden.

Light Exposure

Embryos were exposed to constant cold-light (1800 Lux), constant darkness, or a light-dark shift schedule (12-h light-dark cycle). Under these conditions, zebrafish embryos were raised at identical temperature in all three conditions, i.e., 28.5°C. The embryos were kept at a density of 50 or less in approximately 30 ml medium in a 10 cm bacterial culture plate placed in the running water on each shelf.

Morpholino Treatment

Morpholinos used in this study include include: *bmal1a*-MO (5'-TATCCATTCTTTGGTCTGCCATTAG-3'); *bmal1a*-MIS-MO (5'-TATCGATTGTTTGGTCTGCGATTAG-3'); *bmal2*-MO (5'-CAGATTTCATTCCAGGTGTCCAT-3'); *period2*-MO (5'-GGTCTTCA GACATCGGACTTGGGT-3'); *period2*-MIS-MO (5'-GGTGTTCACAGATCCGACTTCGGT-3'); *clock3*-MO (5'-ATGATAACTCGG TCTCATGGATCA-3'); *vegfaa*-MO (5'-GTATCAATAAACACCAAGTTCAT-3'); *rbpsuh*-MO (5'-CAAACCTCCCTGTGACACA GCGC-3'); and standard scrambled control-MO (5'-CCTCTTACCTCAGTTACAATTATA-3'). These morpholinos have been previously used in other studies (Nasevicius et al., 2000; Siekmann and Lawson, 2007; Triqueneaux et al., 2004; Ziv et al., 2005) and were prepared at 3 mM stocks that were subsequently diluted in nuclease-free water. Approximately 1 nl was injected in the yolk of each embryo at the 1–2 cell stage. The injected embryos were then placed in fresh Danieau's medium or E3 medium, followed by exposure to LD, LL or DD as indicated above.

Assessment of Morpholino Efficiency

Sequences encoding the *bmal1a*, *bmal2*, *period2* and *clock3* open reading frames including the upstream attachment site of each morpholino were amplified from whole zebrafish cDNA using the following primers: zebrafish *bmal1a* Forward: 5'-CACACAGGA TCCTCTGTGCGGATGATCTAATGGC-3' and Reverse: 5'-CACACAGAATTCCAAAGGCCAGGCAGGTCAC-3'; zebrafish *bmal2* Forward: 5'-CACACAGGATCCGGTTTGATGACCCAC-3' and Reverse: 5'-CACACAGAATTCGAGAGACCAGTGCATCTC-3'; zebrafish *period2* Forward: 5'-CACACAGGATCCGGTGATTTGTTCTGTGTTTAC-3' and Reverse: 5'-CACACAGAATTCGGTGTCTG GACCGG-3'; zebrafish *clock3* Forward: 5'-CACACAGGATCCGGCAGGTCCTCTTACC-3' and Reverse: 5'-CACACACTCGAGC TCGAGCTGAGACTGGGCACTAGC-3'. These were cloned into the pCS2-C-Myc vector to generate C-terminal myc-tag fusion constructs via BamHI/EcoRI or BamHI/XhoI sites, respectively. Capped mRNA was generated using the mMessage/Machine kit (Ambion) according to the manufacture's procedure. Each zebrafish embryo was injected at the 1 cell stage with 15 ng capped mRNA in presence or absence of 0.6 mM of the corresponding morpholino in a total volume of 1 nl. Myc-fusion proteins were detected in the presence or absence of morpholino by immunohistochemistry with a 9E10 monoclonal anti-myc antibody (Sigma) in conjunction with NBT/BCIP staining using an alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (Sigma).

Pharmacological Inhibition of VEGFRs or Notch Signaling

ZN323881, sunitinib malate (LC Laboratories, MA) and DAPT (Sigma) were dissolved in DMSO. Embryos exposed to the same concentration of DMSO in the embryo medium were used as a vehicle control.

RT-PCR

Zebrafish embryos (20–50 embryos/group) were dispensed into 1 ml TRIzol (QIAGEN), followed by homogenization for 30 s. Total RNA (1 µg) from each sample was reverse-transcribed using a RevertAid H minus First Strand cDNA Synthesis Kit (Fermentas).

Reverse transcription was performed at 42°C for 60 min, followed by 70°C for 5 min to inactivate the enzyme activity. Samples were stored at –20°C or directly subjected to RT–PCR. RT–PCR was performed in a 20 µl reaction mixture containing DreamTaq Green PCR Master Mix (Thermo Scientific), 500 nM forward and reverse primers, and 1 µl of cDNA. The RT–PCR protocol was executed for 30 cycles and each cycle consisted of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. In the initial cycle, samples were incubated at 95°C for 2 min. The primer pairs specific for various genes used in our experiments are: zebrafish *period2*, Forward: 5′–AAGCCAAGACGCGAGAAAGA–3′, and Reverse: 5′–CAAGAATGCCAAGTTTGTGG–3′; zebrafish *bmal1a* Forward: 5′–GCTGTGGCTCCAGTCCTTTA–3′, and Reverse: 5′–TTCATCATTACTGGGGCTGTGTGA–3′; zebrafish *vegfaa*, Forward: 5′–AGAAAGAAACCAGTGTGAG–3′, and Reverse: 5′–AGGAATGTTCTTCTTAGGT–3′; zebrafish *β-actin*, Forward: 5′–TTGTAACCACTGGGACGATATGG–3′, and Reverse: 5′–GATCTTGATCTTCATGTGTCTAGG–3′.

Zebrafish VEGF-Luciferase Construction and Promoter Deletion

A 2.5 kb fragment (nucleotide position –2500 to –4; *zvegfr* ptr 2.5kb) of the promoter for the zebrafish *vegfab* gene was generated by PCR from zebrafish genomic DNA using the following primers: Forward: 5′–AGCTCGCTTTCTCTGTCATT–3′; and Reverse: 5′–TTAACGAAGTCTTCAGCTG–3′. The promoter fragments of 1090 bp (–1093 to –4; *zvegfr* ptr 1kb) and 857–bp (–860 to –4; *zvegfr* ptr 0.86kb) were amplified from the 2.5 kb fragment using the following paired primers: Forward: 5′–GTTGTGATTTCTCTGGGCTAG–3′; and Reverse: 5′–TTAACGAAGTCTTCAGCTG–3′; and Forward: 5′–GCCGATGATATTCAAACTGG–3′; and Reverse: 5′–TTAACGAAGTCTTCAGCTG–3′; respectively. *zvegfr* ptr 0.86kb Δ was generated by PCR using *zvegfr* ptr 0.86kb as a template and the following mutant synthetic oligonucleotide primers: Forward: 5′–ATAATAAGTTTATTGTTATTGTAAAGCATATGCAAA TGC–3′; and Reverse: 5′–GCATTTGCATATGCTTTACAAATAACAATAAATTATTAT–3′. The above amplified promoter fragments were cloned into the pGL3-Basic vector (Promega, Madison, Wisconsin), according to the manufacturer's instructions, to generate *zvegfr* promoter–luciferase reporter constructs.

Promoter Activity Assay

Zebrafish embryos were injected at the 1–2 cell stage with 1 nl of 100 ng/nl human *vegfr* promoter–luciferase, mouse *bmal1* promoter–luciferase (kindly provided by Dr. Yagita, Nagoya University, Japan), or zebrafish *vegfab* promoter–luciferase, followed by immediate transfer to LD or LL conditions. Five embryos were harvested at each indicated time point from each group and were placed for 30 min in 100 µl of a passive lysis buffer (Vector). The lysed solution was centrifuged at 10 000 rpm for 5 min. 20 µl of the supernatant were added to 100 µl of a luciferase activity reagent from the dual luciferase kit (Vector). The values were recorded from a luminometer and were normalized to the total amount of protein in the sample as determined by the Dc protein assay according to the manufacturer's instructions (BioRad, Sweden).

Quantitative Real-Time PCR

Primer sequences are as follows: zebrafish *vegfaa* Forward: 5′–GAGAGCCAGCGACTCACCGCAACAC–3′; and Reverse: 5′–GTT CGCTCGATCATCATCTTGGC–3′; zebrafish *bmal1a* Forward: 5′–GCAACCGCAAGAGAAAGGCGAGT–3′; and Reverse: 5′–CTCG CTGTTACTGGGGCTGCTTGA–3′; zebrafish *period2* Forward: 5′–AAGCCAAGACGCGAGAAAGAG–3′; and Reverse: 5′–CCACAAA CTGGCATTCTTG–3′; zebrafish *β-actin* Forward: 5′–TTGTAACCAACTGGGACGATATGG–3′; and Reverse: 5′–GATCTTGATCTTCA TGGTGCTAGG–3′.

Chromatin Immunoprecipitation Assay

Mouse fibroblast cells were used for the EZ–ChIP chromatin immunoprecipitation assay (Millipore, Billerica, MA) as previously described (Xue et al., 2012). In brief, 100 µl of sonicated chromatin was used for each immunoprecipitation reaction. A rabbit anti-Bmal1 antibody (2.5 µg per sample, Abcam, Cambridge, UK) and a non-immune rabbit IgG (2.5 µg per sample, Millipore, Billerica, MA) were used for immunoprecipitation. The purified DNA was used for quantitative PCR analysis. To amplify the mouse *vegfr* promoter fragment containing E-boxes 1–3, the following primers were used: Forward: 5′–GTCTACAGCCTCTGTTATGCCAC–3′ and Reverse: 5′–GGTTTCCTCACCTGATCCTG–3′. For amplification of a fragment containing E-boxes 4 and 5, the following primers were used: Forward: 5′–CCGCACTCAAGAATCAACTC–3′ and Reverse: 5′–GCAGGACGTATGAGGATAG–3′. An E-box-less fragment from the mouse *vegfr* gene (4537–4813 downstream from the initial codon) was used as a negative control, which was amplified using primers: Forward: 5′–GTACACTCACACGAGCCATC–3′ and Reverse: 5′–CCCATGACGATGCTTCTCTC–3′. A mouse *period1* fragment, known to bind to Bmal1, was used as a positive control, and this fragment was amplified using primers: Forward: 5′–GCAGTATTGGGTAAGTGTCTGTC–3′ and Reverse: 5′–GCAACCATTTGGCATTTCAGAAC–3′.

Image Analysis

The 4 ISVs posterior to the fusion of two arms of the common cardinal vein into the posterior cardinal vein were measured as mean determinants of ISV length. The SIV with the largest area was used as the SIV area in each embryo. At least 12 embryos were analyzed per group and at least 3 separate experiments were performed. Images were subsequently analyzed in either Photoshop CS4 (Adobe) or ImageJ.

SUPPLEMENTAL REFERENCES

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Figure S1. Knockdown Efficiency of Morpholinos, Related to Figure 2.

(A) Immunohistochemistry of clock gene mRNA-injected zebrafish embryos with or without morpholinos. The translated proteins were detected using an anti-myc tag antibody, followed by detection with an alkaline phosphatase-conjugated secondary antibody. The translated positive signals were revealed using the NBT/BCIP technique. Blue color shows positive signals of the translated proteins.

(B) Quantifications of average clock protein levels $\pm$ SEM in mRNA-injected zebrafish embryos (18-24 embryos/group).

Figure S2. Morphology and Expression Patterns of Sonic Hedgehog and Acetylated Tubulin in Zebrafish Embryos, Related to Figures 1 and 2 Zebrafish embryos at the 1–2 cell stage received injections of specific morpholinos targeting *Bmal1* (B1MO), or *Period2* (P2MO) (0.2 pmol/embryo) and subsequently raised under LL or LD conditions. Scrambled morpholino (scrMO, 0.2 pmol/embryo), 5 base-pair mismatch *bmal1* (B1misMO, 0.2 pmol/embryo), and 5 base-pair mismatch *period2* (P2misMO, 0.2 pmol/embryo) were used as controls. Expression of sonic hedgehog (*shh*) at 24 or 48 hpf was detected by in situ hybridization. Expression of acetylated tubulin (Ac-Tub) was detected by immunohistochemistry using a specific anti Ac-Tub antibody. NC = notochord; EN = enteric nerve.

Figure S3. Additive Effect on Suppression of Developmental Angiogenesis by a Bmal1 Morpholino and Sunitinib, Related to Figure 3
 (A) *Tg(Fli1:egfp)^{y1}* zebrafish embryos were injected with scrambled control (scrMO), *bmal1* (B1MO) or *period2* (P2MO) morpholinos (0.2 pmol/embryo), and placed in medium with either the vehicle buffer or sunitinib. ISV development was investigated at 24 hpf. Scale bar = 50 μ m.
 (B) Quantification of the average ISV length $\pm$ SEM of 24-hpf embryos (n = 4 embryos/group).

Figure S4. RT-PCR Analysis of Circadian Rhythm of Zebrafish *bmal1* and *vegf* Expression, Related to Figure 3

(A) RT-PCR analysis of zebrafish *bmal1* and *vegf* expression under LD conditions (a pooled sample of 25 zebrafish embryos/time point). Standard loading was indicated by β -actin expression.

(B) Densitometric quantification of average $\pm$ SEM *bmal1* and *vegf* expressions shown in (A).